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TITLE:

Analysis of Actomyosin Dynamics at Local Cellular and Tissue Scales Using Time-lapse Movies of Cultured *Drosophila* Egg Chambers

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SUMMARY:

This protocol provides a Fiji-based, user-friendly methodology along with straightforward instructions explaining how to reliably analyze actomyosin behavior in individual cells and curved epithelial tissues. No programming skills are required to follow the tutorial; all steps are performed in a semi-interactive manner using the graphical user interface of Fiji and associated plugins.

ABSTRACT:

Drosophila immature eggs are called egg chambers, and their structure resembles primitive organs that undergo morphological changes from a round to an ellipsoid shape during development. This developmental process is called oogenesis and is crucial to generating functional mature eggs to secure the next fly generation. For these reasons, egg chambers have served as an ideal and relevant model to understand animal organ development.

Several in vitro culturing protocols have been developed, but there are several disadvantages to these protocols. One involves the application of various covers that exert an artificial pressure on the imaged egg chambers in order to immobilize them and to increase the imaged acquisition plane of the circumferential surface of the analyzed egg chambers. Such an approach may

negatively influence the behavior of the thin actomyosin machinery that generates the power to rotate egg chambers around their longer axis.

Thus, to overcome this limitation, we culture *Drosophila* egg chambers freely in the media in order to reliably analyze actomyosin machinery along the circumference of egg chambers. In the first part of the protocol, we provide a manual detailing how to analyze the actomyosin machinery in a limited acquisition plane at the local cellular scale (up to 15 cells). In the second part of the protocol, we provide users with a new Fiji-based plugin that allows the simple extraction of a defined thin layer of the egg chambers' circumferential surface. The following protocol then describes how to analyze actomyosin signals at the tissue scale (>50 cells). Finally, we pinpoint the limitations of these approaches at both the local cellular and tissue scales and discuss its potential future development and possible applications.

INTRODUCTION:

The continual development of novel imaging and software technologies with applications in the life sciences has provided an enormous impact on understanding the basic principles of life. One of the main challenges is the reliable visualization of developmental processes in combination with their live imaging in various tissues. Tissues are parts of organs and bodies and, as such, the majority are not easily accessible for imaging. Therefore, protocols that allow their dissection and in vitro culturing have been developed in order to visualize biological events that sufficiently reflect the in vivo situation within a living body.

Over the past decades, the culturing and live imaging of *Drosophila* egg chambers, acinar-like structures resembling primitive organs, has contributed immensely to the understanding of the basic principles of primitive organ development[1–3]. Currently, there are several culturing protocols available, and their usage depends on acquisition time, cell type to be imaged, and their accessibility (e.g., inner germline vs. outer somatic line)[4].

A common feature in all these culturing protocols is the need for the immobilization of analyzed egg chambers that display a high contractile activity in liquid media. The contractile activity of egg chambers is caused mainly by the muscle sheet that covers a long string of connected egg chambers[5–7]. Therefore, to achieve proper immobilization of young egg chambers, various approaches have been developed, involving covering egg chambers with coverslips[6,8,9] or flexible blankets[4,10] or embedding them in low-melting-point agarose[3,11]. These approaches are popular as they also allow the imaging of a larger visual plane due to the subtle flattening of the circumferential surface of the egg chambers.

However, recently, it has been shown that young egg chambers (stage 1–8) rotate around their anterior-posterior axis[6] and that this tissue motion is powered by a fine actomyosin network close to the circumferential surface of these young egg chambers[12]. Therefore, artificial alteration of the cellular surface caused by a subtle flattening of this tissue may have a negative impact on the behavior of the force-generating actomyosin machinery. The counterpoint is that if the egg chamber tissue is not flattened, microscopic imaging of proteins at the circumferential surface of egg chambers becomes even more limited by the decreased size of the acquisition plane.

Therefore, we have combined protocols from Prasad et al.[9] and the lab of Celeste Berg[4,10] and further modified them so that no coverslip/flexible blanket/agarose is used in the developed method. *Drosophila* egg chambers are freely cultured in media and the protocol presented here applies only inverted microscopy. There are two parts to the protocol. The first part is focused on the analysis of actomyosin signals at the local cellular scale (up to 15 cells) within egg chambers. In the second part, we focus on overcoming the limitations associated with a small acquisition plane caused by the free culturing of egg chambers. In this regard, we have developed a novel Fiji-based computational method with a semi-interactive graphical user interface that selectively extracts and unfolds defined layers of a circumferential tissue surface. This is followed by a protocol that describes how to analyze actomyosin at the tissue scale (i.e., >50 cells). As the selective extraction of a defined thin layer of curved epithelial tissues has not been easily possible using a classical z-stack projection (**Figure 1**), this easy-to-use method serves as an important prerequisite to comprehensively understanding the behavior of a thin (<1 µm) actomyosin network at the tissue scale in *Drosophila* egg chambers.

In addition, to facilitate the protocol, we provide example time-lapse movies (TLMs) and sample files of fluorescently tagged nonmuscle conventional myosin II behavior (see **Supplementary File 3**). Myosin II is a motor protein and represents the active contractile part of the actomyosin machinery. In order to image myosin II, we use *Drosophila* transgenic lines that contain a modified regulatory light chain of myosin II called MRLC::GFP (see **Table of Materials** for details)[12,13]. In order to visualize cell membranes, the protocol is based on commercial dyes (see **Table of Materials**). This protocol is suitable not only for the analysis of small subcellular MRLC::GFP signals[12] but also for any similar-sized subcellular particles around ±300 µM, such as those observed with Life-Act::GFP[12,14].

Although both these protocols are presented using in vitro cultured *Drosophila* egg chambers, the acquisition of actomyosin signals can also be performed using other tissues upon the optimization of the culturing media and depending on the availability of either fluorescently tagged proteins with corresponding commercial dyes or, for example, mRNA microinjections to obtain transient gene expression profiles. Similarly, the Fiji-based protocol for the extraction of a thin layer from a circumferential surface can be applied more generally to ellipsoid and organ-like tissues.

PROTOCOL:

NOTE: The following protocol provides instructions on how to analyze actomyosin at the local cellular and the tissue scale in *Drosophila* egg chambers. The local-scale approach allows users to analyze detailed actomyosin behavior in up to 15 cells per egg chamber and requires the acquisition of TLMs for a short period of time (5–10 min) by using high-speed imaging and an inverted confocal microscope. In contrast, the tissue scale provides users with actomyosin information in 50–100 cells and requires the acquisition of TLMs for a long period of time (≥30 min) by using low-speed imaging and an inverted spinning disc microscope (see **Figure 2** and recommended parameters at each scale in **Table 1**). The decision at which scale to analyze

actomyosin signals entirely depends on the user's scientific question. Accompanied test TLMs should help to make this decision.

1. Local cellular scale (LCS)

NOTE: To dissect and image in vitro cultured *Drosophila* egg chambers, follow the protocol described in **Supplementary File 1**. To analyze acquired TLMs, continue with the following protocol. Links to accompanying test files of TLMs are provided in the **Supplementary File 3**.

1.1. Data processing of TLMs at the local cellular scale (LCS)

1.1.1. Bleach correction of TLMs to compensate for intensity decay of fluorescent labels

1.1.1.1. Make sure an up-to-date Fiji application is installed on the computer being used by following these instructions: **Fiji** > **Open a TLM** (e.g., TestMovie1.tif from the **Supplementary File 3**).

1.1.1.2. Split the color channels by clicking **Image** > **Color** > **Split Channels**.

1.1.1.3. Perform a bleach correction on both channels using **Image** > **Adjust** > **Bleach Correction** > **Simple Ratio** > **Background Intensity 0.0**.

NOTE: If unsatisfactory results are obtained, explore which correction methods in this plugin fits best (e.g., use **Histogram Matching** instead of a **Simple Ratio**).

1.1.2. Cell segmentation to generate a cell mask for TLMs

1.1.2.1. If Fiji is not already open, reopen it (and make sure it is up-to-date) following **Help** > **Update** > **Apply Changes** > **OK**.

1.1.2.2. If this has not already been done, download the macro **TissueCellSegmentMovie.ijm** (from http://adm.irbbarcelona.org/matlab/TissueCellSegmentMovie.ijm). Drag and drop this script into Fiji.

1.1.2.3. Open the bleach-corrected TLM .tiff file (see section 1.1.1). Split the channels of the bleach-corrected file using **Image** > **Color** > **Split Channels**.

1.1.2.4. Run the uploaded script on the active cell membrane channel of the selected TLM by pressing the icon **Run** in the open script. Set the Gaussian blur to **1.500** and the cell detection sensitivity to **-1** and click **OK**. In order to get a nice cell mask, do not change these parameters above. Set the estimated noise tolerance between 10–20 for TLMs acquired with the 63x objective and click **OK**.

NOTE: For other objectives, different parameters may be required. The cleaner the background in a TLM, the lower **Estimated noise tolerance** required.

1.1.2.5. A generated cell mask appears in the analyzed TLM and also in a new window called **ParticleStack (G)**, and in addition, a little window called **Action Required** appears. From the cell mask on the TLM, focus only on cells in the center of the TLM and select those that can provide complete and well-defined outlines throughout the TLM.

1.1.2.6. If selected cells contain artificial/extra cell outlines, use the merge tool window called **Action Required** in the TLM. For the latter, follow the instructions in the window.

NOTE: Make sure that cell outlines are well-defined and correspond to real cell membranes in a TLM as any imprecision may impact subsequent analyses. Additional tweaks and changes, such as the removal of unwanted cells, can be done later using the Surface manager tool (described in section 1.1.3).

1.1.2.7. When the selected cells in the center nicely correspond to the real cell membranes, save ParticleStack as a .tiff file following **File** > **Save As** > **Tiff**.

1.1.3. Loading of the generated cell mask into Surface manager

1.1.3.1. Install Surface Manager Plugin here[15] into the used Fiji setup. Follow the instructions by Viktorinova et al.[16].

1.1.3.2. Open Surface Manager using **Plugins** > **Segmentation** > **Surface manager(3D).**

NOTE: The Surface manager window displays several action buttons on the right and an empty window on the left. To see all action buttons, it may be required to stretch the window in its height/width. Note that time frames will appear as z-slices.

1.1.3.3. Open the corresponding ParticleStack .tiff file into Surface Manager by clicking **File** > **Open** and select the image to open (e.g., ParticlesStack1.tif).

1.1.3.4. In Surface Manager, click the **Read outline image** button and set the Jacquard index to 60%.

NOTE: Loading the ParticlesStack1.tif file can take up to several minutes, depending on the computer's processing power.

1.1.3.5. Once loaded, each cell will be assigned with an S number and appear in the left part of the Surface manager window.

NOTE: To show outlines and cell names for all cells, tick the **Show all** and **Show labels** checkboxes at the bottom of the Surface manager window. It is recommended to use this function once the cell numbers have been checked for their correctness.

1.1.3.6. Click on the first S number; the imported cell outline from ParticlesStack1.tif appears on the TLM. Check each imported S number for cell outline quality throughout the TLM and remove unwanted cells that display incorrect cell outlines. To do so, highlight the cell outline and click on the button **Delete**.

NOTE: It is also possible to correct for imprecise cell outlines and add new cell outlines. This is described in the discussion section.

1.1.3.7. Save all corrected cells as a RoiSet.zip file by pressing the **Save to disk** button.

NOTE: If the session needs to be interrupted, it is possible to load the RoiSet file into Surface manager later: open a TLM in Fiji (**File** > **Open**) and select a TLM. Open Surface manager via **Plugins** > **Segmentation** > **Surface manager(3D)**. Load the corresponding RoiSet.zip file by clicking the **Load from disc** button and choosing the appropriate RoiSet.zip file. Double-check that the loaded cell masks correspond to the loaded TLM.

1.1.4. Correction for tissue drift in TLMs

NOTE: During TLMs' acquisition time, drift effects may be observed due to epithelial rotation or an unwanted movement. In both cases, we recommend correcting for any drift in order to simplify the manual actomyosin analysis later. Drift correction is required only for the manual actomyosin analysis. This tutorial requires up-to-date Fiji software with the MultiStackReg Plugin (https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Software/Software_installation.md#multistackreg) installed.

1.1.4.1. Open the bleach-corrected TLM in Fiji via **File** > **Open** and select the bleach-corrected file created using the tutorial mentioned above..

1.1.4.2. Split the channels and select the one that identifies the cell membranes via **Image** > **Color** > **Split Channels**.

1.1.4.3. Use the following protocol when the cell outlines are not visibly smooth: **Process** > **Filters** > **Gaussian Blur**. Set it to ~1–2 for a 63x objective and click **OK** and then **Yes**. Click **Process** > **Binary** > **Convert to Mask** using the default settings; then, click **OK**.

1.1.4.4. Load the MultiStackReg Plugin following **Plugins** > **Registration** > **MultiStackReg**.

1.1.4.5. Make sure Stack 1 is set to the cell membrane channel, Action 1 is set to Align, and Transformation is set to Translation. Check the **Save Transformation File** checkbox and then click **OK**.

1.1.4.6. Reopen the selected TLM, the MultiStackReg Plugin, and the saved transformation file using **File** > **Open** and select a TLM. Split the color channels using **Image** > **Color** > **Split channels**.

1.1.4.7. Load the MultiStackReg Plugin by clicking **Plugins** > **Registration** > **MultiStackReg**. Select Load Transformation File as Action 1.

1.1.4.8. Leave Transformation as **Rigid Bod** and click **OK**. Select the previously saved Transformation File and click **OK** again.

1.1.4.9. Merge the image channels and save the registered TLM as a .tiff file following **Image** > **Color** > **Merge channels**. Save the file by clicking **File** > **Save As** > **Tiff**.

NOTE: There are alternative ways to correct for a tissue drift in Fiji, namely via **Plugins** > **Registration** > **StackReg/Correct 3D drift**.

1.2. Analysis of actomyosin pulses in Surface manager at LCS

NOTE: This protocol step allows scientists to identify whether actomyosin pulses are present in the analyzed tissue and to understand the detailed behavior as well as the directionality of actomyosin signals.

1.2.1. Open Fiji and ensure that a TLM and its corresponding cell mask is open in Surface manager.

NOTE: Ensure Fiji is installed and up-to-date and the Surface Manager Plugin is installed (step 1.1.3.1).

1.2.2. Open a TLM with **File** > **Open** and select a TLM to open (e.g., TestMovie1_bleach.tif). Load the Surface manager Plugin via **Plugins** > **Segmentation** > **Surface manager(3D)**. Load the saved cell mask created in the tutorial here (e.g., ParticlesStack1.tif) via **File** > **Open** and select a cell mask (e.g., ParticlesStack1.tif). Load the corresponding region of interest (ROI, e.g., TestMovie1_RoiSet.zip). See **Figure 3A**.

1.2.3. Switch to the channel of interest in the selected TLM.

NOTE: To distinguish the channels, follow the color code indicated around the TLM.

1.2.4. In Surface Manager, click the **Statistics** button to obtain the window called **Average grey value Slice by Slice** (**Figure 3B**). Note that the mean/median intensity values of actomyosin signals in a given analyzed channel within the defined cell outlines over time will be displayed, as well as other parameters related to the cell area and cell shape.

NOTE: The intensity values are in arbitraty units (A.U.); the cell area is in pixels and needs to be converted into square micrometers.

1.2.5. Save the obtained values as a spreadsheet file. Click on the Statistics window, **File** > **Save As**.

NOTE: It is possible to verify obtained statistical values later as follows: open the bleach-corrected TLM in Fiji and open Surface manager. Load the corresponding RoiSet.zip to the TLM by pressing the **Load from disk** button and open the file. The saved mask with cell outlines will be uploaded and the names of the cells will appear in the left Surface manager window. Click the **Statistics** button for statistical values.

1.3. Manual analysis of individual subcellular actomyosin signals at LCS

NOTE: This protocol requires the most concentration and is time-consuming. In general, one acquired TLM can be comfortably analyzed within 1 day. This does not include time for fly preparation before their dissection, the dissection itself, and image acquisition.

1.3.1. Open a selected, bleach-corrected and registered (drift-corrected) TLM in an up-to-date Fiji application. Use a bleach-corrected and drift-corrected TLM (e.g., TestMovie1_bleach_reg.tif).

1.3.2. Adjust the brightness and contrast to see the individual actomyosin signals using **Image** > **Adjust** > **Brightness/Contrast**.

1.3.3. Align TLMs in the same direction relative to the tissue/body axis. In the case of egg chambers, align the anterior side of the egg chambers always to the left of the image/submovie/TLM before the analysis.

1.3.4. Divide the selected movie into short 30 s submovies and time-project each of them. Save nontime-projected and time-projected submovies with corresponding names. Keep the original source.

NOTE: Submovies can be created from original TLMs by first deleting unwanted frames via **Image** > **Stacks** > **Slice Remover**. Define the slices to be removed and set Increment. Transform to RGB before using the slice remover when Increment = 1. To time-project a 30 s submovie, click **Image** > **Stacks** > **Z-project** > **Max Intensity** for the defined frames (slices) and then click **OK**. Skip every second 30 s submovie in order to avoid counting actomyosin signals 2x in two subsequent 30 s submovies.

1.3.5. Place a time-projected image next to the corresponding 30 s submovie (**Figure 4A,B**). Identify the signal line on the time-projected submovie. Identify the direction of the actomyosin signal movement (0°–360°) along this line in the original submovie by manually playing the

submovie. Use the **Angle** tool (in the Fiji bar) to manually measure the direction of the signal movement relative to the defined tissue axis or a similar available tool.

NOTE: Fiji works only on a 0°–180° scale (**Figure 4C**), and a recalculation of the obtained values onto a 0°–360° scale is required. A signal line corresponds to the trajectory of one actomyosin signal movement over time.

1.3.6. To avoid duplication in the analysis of actomyosin signals, mark the analyzed signal lines within cells in the time-projected submovie (**Figure 4B,D**).

1.3.7. Analyze all selected cells in the 30 s submovie and save the obtained angles.

NOTE: Analyze only subcellular cytoplasmic actomyosin signals in cells with well-defined cell outlines throughout a TLM. Exclude individual cells with less than 15–20 detected signals in the whole TLM. Ignore actomyosin signals that move across cells due to their unknown origin. An example of signal counts for one analyzed cell in the 30 s submovie is shown in **Figure 4D**.

1.3.8. Continue until all 30 s-long submovies of one TLM are analyzed, and save all measured angles of actomyosin signals of one TLM in a spreadsheet file.

1.3.9. Sum up all measured angles over the relevant number of TLMs (dependent on the experiment). Plot the percentage of the direction of analyzed actomyosin signals, for example, as a rose diagram with a range from 0° to 360° with a preferred software.

NOTE: To obtain statistically significant results, five to ten independent egg chambers of any egg chamber stage are recommended to be analyzed.

2. Tissue scale

NOTE: To dissect and image in vitro cultured *Drosophila* egg chambers, follow the protocol in **Supplementary File 2**. To analyze acquired TLMs, continue with the following protocol below. Accompanied test files of TLMs are placed in the **Supplementary File 3**.

2.1. Selective surface extraction of curved epithelial tissues

NOTE: This protocol allows users to selectively extract a thin layer of actomyosin in a curved epithelial tissue over time. This protocol step is user-friendly and based on an intuitive graphical interface. It is possible to comfortably analyze (extract surface) several TLMs. Use TestMovie2.czi (from the **Supplementary File 3**) as a test TLM example.

2.1.1. Open an up-to-date Fiji application (**Fiji** > **Help** > **Update** > **Apply Changes** > **OK**).

2.1.2. Ensure the Ellipsoid Surface Projection plugin[15] is installed. Follow the instructions by Viktorinova et al.[16].

2.1.3. Open a TLM and export it as an XML/HDF5 file by clicking **Plugins** > **BigDataViewer** > **Export Current Image as XML/HDF5 file**.

NOTE: It is required to define an export path. The saving itself can take several minutes and depends on the TLM length.

2.1.4. Open the exported file in Ellipsoid Surface Projection by clicking **Plugins** > **BigDataViewer** > **Ellipsoid Surface Projection** > **Select XML file**.

NOTE: A new window with sagittal views of the egg chamber and a dialog to guide the user through the processing will appear. Details on how to navigate in the slice view can be found at https://imagej.net/BigDataViewer[17].

2.1.5. The dialog window called Ovaries Projection has several tabs. In the first dialog tab called Bounding box, define the x and y borders of an egg chamber in the TLM together with the z width of the bounding box (i.e., the depth of a z-stack/egg chamber) and press **set** (**Figure 5A**).

2.1.6. To define borders, drag buttons next to x, y, and z or define the number coordinates in the x, y, and z boxes. Ensure that the borders are generous enough to get the part of the egg chamber of interest into the surface extraction. The selected parts of the egg chamber are highlighted in pink.

2.1.7. Switch to the tab called **Find blobs** in the window Ovaries Projection. Define the sigma and the minimal peak value; then, press compute to identify the actomyosin signals (**Figure 5B**), which will appear as green blobs. Retry this step with different parameters until enough spots (around 100) are found.

NOTE: Sigma 1–3 with minimal peak value 20–100 works the best to identify actomyosin signals of stage-6 to -8 egg chambers. Identifying actomyosin signals is a crucial step and depends on the signal quality and its size. It is important to confirm the correct size of the existing blobs. No visible green spots/blobs in the image means that the next step will not work. Browse through the selected z planes to see blobs.

2.1.8. To design the ellipsoid, continue with the tab called **Fit ellipsoid**. Set **Random samples** to 10,000, **Outside/inside cut-off distance** to 1–10, and then, click **Compute** (**Figure 5C**).

NOTE: The lower the cut-off distance, the more precise the desired ellipsoid will be. After this, the tab called **Projection** will automatically open and allows the definition of the surface extraction. The settings need to be optimized.

2.1.9. Continue further with the tab called **Projection** (**Figure 5D**). Set up a minimum and maximum projection distance (i.e., the width of the desired ellipsoid). It is required to set a

minimal and maximal projection distance so that the pink defined ellipsoid region includes the entire outside layer of the egg chamber. Define the slice distance (1 is recommended).

NOTE: Examples of an incorrect and an optimal ellipsoid fit resulting in incorrect and optimal projection parameters are shown in **Figure 6A,C**. The thin circumferential layer of the interest can be defined later. Make sure that the pink region of the ellipsoid with the defined width on the egg chamber is visible before pressing compute. It is important that the desired ellipsoid (pink single line) nicely fits the ellipsoid surface of an egg chamber in order to obtain the best results. If the ellipsoid fit is not good, arrange different settings till the desired surface extraction is obtained.

2.1.9.1. Set an output width (≥800) and height (≥400) of the ellipsoid. Set from which time point to which time point the surface extraction should be created (i.e., define the length of the TLM extraction). Choose either a spherical or a cylindrical projection. Flip Z and align Y if required.

2.1.9.2. Press compute to obtain a surface extraction for both channels in new windows called **Image**. Adjust the brightness and contrast of the obtained image windows to be able to see the projected actomyosin signals, by clicking **Image** > **Adjust** > **Brightness/Contrast.**

NOTE: An example comparison of a projection resulting from an incorrect and optimal ellipsoid fit is shown in **Figure 6B,D**.

2.1.10. If the surface extraction looks good, save the images (both channels separately) as .tiff. Additionally, save the Log window file for future reference as to how the surface of this particular egg chamber was extracted.

NOTE: This is particularly important information if the extracted thin layer should be returned to its original unfolded shape (for developers only).

2.1.11. Merge the channels with a preferred color code in Fiji.

NOTE: If necessary, project selected z-stack layers with actomyosin signals. The projection of selected z-stack layers is required when the acquisition focus slightly changes over time in a TLM. For best results, project at most one to two z-stack layers.

2.1.12. Save the results as .tiff files.

NOTE: Representative examples of thin layers (selective basal and apical surface) that represent the myosin II (MRLC::GFP) signal from the follicle epithelium of the control and *fat2* mutant egg chambers can be found in **Figure 1**.

2.2. Data processing of a selectively extracted tissue surface

2.2.1. Bleach-correct the TLMs to compensate for intensity decay of the fluorescent labels.

2.2.1.1. Open Fiji. Open a TLM (e.g., TestMovie2.czi from the **Supplementary File 3**) using **File** > **Open**. Select the image to open it.

2.2.1.2. Split the color channels and convert them to 16-bit images, if necessary, by clicking **Image** > **Color** > **Split Channels**. Convert the channels to 16-bit images (from 32-bit images) using **Image** > **Type** > **16-bit**.

2.2.1.3. Perform a bleach correction on both channels using **Image** > **Adjust** > **Bleach Correction** > **Simple Ratio** > **Background Intensity 0.0**.

NOTE: If unsatisfactory results are obtained, it is required to explore which correction methods in this plugin fit best (e.g., use Histogram Matching instead of a Simple Ratio).

2.2.1.4. Merge the channels from the two bleach-corrected images by clicking **Image** > **Color** > **Merge Channels**. Save the merged image as a .tiff file by clicking **File** > **Save As** > **Tiff**.

NOTE: Adjust the contrast and brightness for the channels if needed.

2.2.2. Cell segmentation to generate a cell mask for TLMs

2.2.2.1. If Fiji is not already open, reopen it (and make sure it is up-to-date by clicking **Help** > **Update** > **Apply Changes** > **OK**).

2.2.2.2. If this is not already done so, download the macro TissueCellSegmentMovie.ijm (http://adm.irbbarcelona.org/matlab/TissueCellSegmentMovie.ijm).

2.2.2.3. Drag and drop this script into Fiji. Open the bleach-corrected file TestMovie2_bleach.tif (see step 2.2.1).

2.2.2.4. Split the channels of the bleach-corrected file by clicking **Image** > **Color** > **Split Channels**. Adjust the membrane channel to ensure clear cell outlines by clicking **Image** > **Adjust** > **Brightness/Contrast**.

2.2.2.5. Run the uploaded script on the active cell membrane channel of the selected TLM by pressing the icon **Run** in the open script. Set Gaussian blur to 1.500. Set cell detection sensitivity to -1 and click **OK**. Set **Estimated noise tolerance** to ~8 for TLMs acquired with the 40x objective and click **OK**.

NOTE: In order to get a nice cell mask, do not change these parameters above. For other objectives, different parameters may be required. The cleaner the background in a TLM, the lower **Estimated noise tolerance** is required.

2.2.2.6. A generated cell mask appears in the analyzed TLM and also in a new window called **ParticleStack (G)**. Additionally, a little window called **Action required** appears. From the cell mask on the TLM, focus only on cells in the center of the TLM and select those that can provide complete and well-defined outlines throughout the TLM.

2.2.2.7. If selected cells contain artificial/extra cell outlines, use the merge tool window called **Action required** in the TLM. For the latter, follow the instructions in the window.

NOTE: Make sure that cell outlines are well-defined and correspond to real cell membranes in a TLM as any imprecision may impact subsequent analyses. Additional tweaks and changes, such as the removal of unwanted cells, can be done later using the Surface manager tool (outlined in the tutorial below).

2.2.2.8. When selected cells in the center nicely correspond to the real cell membranes, save the ParticleStack as a .tiff file following **File** > **Save As** > **Tiff**. Proceed to perform the loading and analysis of the generated cell mask in Surface Manager as previously performed in sections 1.1.3 and 1.2 (also described in detail in sections 2.2.3 and 2.3).

2.2.3. Loading of the generated cell mask into Surface manager

2.2.3.1. If the Surface Manager Plugin is already installed in the Fiji setup used, proceed to step 2. If not, follow the instructions by Viktorinova et al.[16]. For developers, just the source code is also available[15].

2.2.3.2. Open Surface Manager by clicking **Plugins** > **Segmentation** > **Surface manager(3D)**.

NOTE: The Surface manager window displays several action buttons on the right and an empty window on the left. To see all action buttons, it may be required to stretch the window in its height/width. Note that time frames will appear as z-slices.

2.2.3.3. Open the corresponding ParticleStack.tif file into Surface Manager via **File** > **Open** and select the image to open (e.g., ParticleStack.tif).

2.2.3.4. In Surface Manager, click the **Read outline image** button and set the Jacquard index to 60%.

NOTE: Loading a ParticleStack.tif file can take up to several minutes, depending on the computer power.

2.2.3.5. Once loaded, each cell will be assigned with an S number and will appear in the left part of the Surface manager window (**Figure 7A**).

NOTE: To show outlines and cell names for all cells, tick the **Show all** and **Show labels** checkboxes at the bottom of the Surface manager window. It is recommended to use this function once the cell numbers have been checked for their correctness.

2.2.3.6. Click on the first S number; the imported cell outline from ParticleStack.tif appears on the TLM. Check each imported S number for cell outline quality throughout the TLM and remove unwanted cells that display incorrect cell outlines. To do so, highlight the cell outline and click on the button **Delete**.

NOTE: It is also possible to correct for imprecise cell outlines and add new cell outlines. This is described in the discussion section.

2.2.3.7. Save all corrected cells as a RoiSet.zip file by pressing the **Save to disk** button.

NOTE: If the session needs to be interrupted, it is possible to load the RoiSet.zip file into Surface manager later: open a TLM in Fiji via **File** > **Open** and select a **TLM**. Open Surface manager as follows: **Plugins** > **Segmentation** > **Surface manager(3D)**. Load the corresponding RoiSet.zip by clicking the **Load from disc** button and choosing the appropriate RoiSet.zip file (e.g., TestMovie2_RoiSet.zip). Double-check that the loaded cell masks correspond to the loaded TLM.

2.3. Analysis of actomyosin pulses in Surface manager

NOTE: Open Fiji and ensure that a TLM and its corresponding cell mask is open in Surface manager. Ensure Fiji is installed and up-to-date and the Surface Manager Plugin (https://git.mpi-cbg.de/tomancaklab/surface_manager) is installed.

2.3.1. Open a TLM with **File** > **Open** and select a TLM to open (e.g., TestMovie2_bleach.tif). Load the Surface manager plugin via **Plugins** > **Segmentation** > **Surface manager(3D)**. Load the saved cell mask created in the tutorial here (e.g., ParticlesStack2.tif) via **File** > **Open** and select a cell mask (e.g., ParticlesStack2.tif). Load the corresponding ROI (e.g., TestMovie2_RoiSet.zip).

2.3.2. Switch to the channel of interest in the selected TLM.

NOTE: To distinguish between the channels, follow the color code indicated around the TLM.

2.3.3. In Surface manager, click the **Statistics** button to obtain the window called **Average grey value Slice by Slice** (**Figure 7B**). Note that the mean/median intensity values of actomyosin signals in a given analyzed channel within the defined cell outlines over time will be displayed, as well as other parameters related to the cell area and cell shape.

NOTE: The intensity values are in A.U.; the cell area is in pixels and needs to be converted into square micrometers.

2.3.4. Save the obtained values as a spreadsheet file: click on the Statistics window, **File** > **Save As**.

NOTE: It is possible to verify the obtained statistical values later, as follows. Open the bleach-corrected TLM in Fiji. Open Surface manager. Load the corresponding RoiSet.zip to the TLM by pressing the **Load from disk** button and open the file. The saved mask with cell outlines will be uploaded and the names of the cells will appear in the left Surface manager window. Click the **Statistics** button for statistical values.

NOTE: Regarding a **manual analysis of individual subcellular actomyosin signals**, it is not possible to perform a manual analysis of subcellular actomyosin signals at the tissue scale. Optimization is required for the analysis of individual actomyosin signals at the semi-tissue scale, as highlighted in the discussion section.

REPRESENTATIVE RESULTS:

This protocol enables scientists to investigate the behavior of actomyosin networks in epithelial tissues. This is only possible when a detailed analysis of actomyosin behavior at the local cellular scale (a few cells) is combined with a similar analysis at the tissue scale (many cells). However, epithelial tissues are often curved and the extraction of a thin layer of these tissues was previously not easily possible, as shown in *Drosophila* egg chambers (**Figure 1**). The protocol presented here provides users with simple instructions describing how to analyze actomyosin behavior at both these scales (**Figure 2**).

The first part of this protocol focuses on instructions regarding the analysis of actomyosin pulses using the **Surface manager** plugin (**Figure 3**). It also describes how to manually analyze individual actomyosin behavior at the local cellular scale (**Figure 4**). The second part of this protocol explains how to extract a thin layer of epithelial tissue using the **Ellipsoid Surface Projection** plugin (**Figure 5** and **Figure 6**). Only then is it possible to analyze actomyosin pulses at the tissue scale using the **Surface manager** plugin (**Figure 7**). Representative results and a comparison of actomyosin behavior in rotating control and static *fat2* mutant *Drosophila* egg chambers at the local cellular and tissue scale is shown in **Figure 8** and in corresponding **Movie 1**, **Movie 2**, **Movie 3**, **Movie 4**, **Movie 5**, and **Movie 6** (for details, see **Figure 8**).

FIGURE LEGENDS:

Figure 1: Selective extraction of a thin layer of a curved tissue surface. (**A**) In order to obtain sufficient information regarding actomyosin networks in circumferentially curved epithelia, it is necessary to acquire a deep z-stack (pink) over the majority of a visible tissue as shown for the curved follicle epithelium (grey) of *Drosophila* egg chambers. (**A'**) However, a simple projection of such a z-stack leads to the mixing of whole actomyosin networks in follicle cells. Myosin II visualized with MRLC::GFP (green) shows the strongly expressing inner (apical) region of follicle cells in such a z-projection and almost no information from the basal (outer) side, where myosin II displays a strong planar cell polarity phenotype, but its signal intensity is low[12]. To avoid such mixing as shown in panel **A'**, we have developed a user-friendly, Fiji-based plugin called **Ellipsoid**

Surface Projection in BigDataViewer[18] that allows (**B**) the selective extraction of a defined, thin layer (pink) of actomyosin from a curved epithelium (**B'**) as shown for myosin II (green) in a selected extraction of the basal (outer) side of the follicle epithelium. Compare the difference in signal of myosin II (MRLC::GFP) in panels **A'** and **B'**. Note the planar polarized pattern of myosin II in panel **B'**. The cell outlines are in red. The anterior side is on the left. The scale bar = 50 µm.

Figure 2: Planar polarized actomyosin network at the local cellular and tissue scales. The imaging of actomyosin at different scales allows the analysis of different numbers of epithelial cells, namely (**A**) up to 15 cells at the local cellular scale and (**B**) 50–100 cells at the tissue scale in the follicle epithelium of cultured *Drosophila* egg chambers. Nonmuscle myosin II is visualized with MRLC::GFP (green) and the genotype of the used transgenic line is specified in the **Table of Materials**. The cell outlines are in magenta. The anterior side is on the left. The scale bars = 5 µm in panel A and 50 µm in panel B.

Figure 3: An example of the analysis of myosin II pulses in Surface manager at the local cellular scale. (**A**) A particle stack is loaded into Surface manager. Note that all identified cells in the TLM appear in the Surface manager window. It is important to delete all unwanted or incomplete cells throughout a TLM. (**B**) Statistics obtained on myosin II (MRLC::GFP) for selected cells are shown in the window called **Average grey value Slice by Slice** in Surface manager. MRLC::GFP is shown in green whilst cellular membranes are in red. The anterior side is on the left.

Figure 4: An example of the manual analysis of individual myosin II signals at the local cellular scale. (**A**) A selected 30 s-long submovie is placed next to (**B**) its time projection. Note that upon the time projection, myosin II (MRLC::GFP) shows longer trajectory lines (see panel **B**) within individual cells than in an individual time frame (see panel **A**). Individual lines in cells should be analyzed for their angular direction relative to the anterior-posterior axis of the egg chambers. (**C**) Note that Fiji does not measure 0° to 360° but only 0° to 180° for signals pointing up and 0° to -179° for signals pointing down. Be aware that 0° is atypically placed on the left of an analyzed image. (**D**) Based on this information, lines that move with (up in pink) or against (down in yellow) the direction of the epithelial rotation in a particular egg chamber should be binned to identify whether symmetry breaking of analyzed signals is present within a cell and then for multiple cells in the analyzed tissue. MRLC::GFP is shown in green whilst cellular membranes are in red. The anterior side is on the left.

Figure 5: An example of generating an ellipsoid fit at the tissue scale. In order to fit an ellipsoid onto an egg chamber using the **Ellipsoid Surface Projection** plugin in BigDataViewer, it is required to define (**A**) a bounding box that includes the majority of the scanned tissue. Next, it is important (**B**) to identify signal dots, which are a prerequisite for an optimal ellipsoid fit generation. (**C**) When the ellipsoid fit is not optimal and does not nicely surround an egg chamber, this results in (**D**) poor tissue layer extraction. See the extraction and later projection results in **Figure 6**.

Figure 6: Comparison of an incorrect and optimal ellipsoid fit and corresponding surface projections. (**A**) When the ellipsoid is not fitted properly, (**B**) the final surface projection will not provide complete signal data in the thin layer of the analyzed tissue. (**C**) However, when fitted

optimally, it guarantees an equal signal detection of a thin layer in the tissue. (**D**) Note that the optimal surface projection contains several thin layers as a surface-projected *z*-stack over time, which can be separated subsequently as shown in **Figure 8**. Myosin II signals (MRLC::GFP, white) and membrane signals (white) are initially merged before the projection (panels **A** and **C**), but upon the surface projection itself, these channels are separated (see projections of myosin II in panels **B** and **D**).

Figure 7: An example of the analysis of myosin II pulses in Surface manager at the tissue scale. (**A**) A particle stack is loaded into Surface manager. Note that all identified cells in the TLM appear in the Surface manager window. It is important to delete all unwanted or incomplete cells throughout a TLM. (**B**) Statistics obtained on myosin II (MRLC::GFP) pulses (mean or median) for selected cells over time are shown in the window called **Average grey value Slice by Slice** in Surface manager. Note that stronger myosin II dots may influence the final measure of intrinsic myosin II intensity. This results from the issue that these myosin II dots may appear to migrate beyond the cell outline where there is an incorrect cell outline definition in the generated cell mask. MRLC::GFP is shown in green whilst cellular membranes are in red. The anterior side is on the top.

Figure 8: Representative results of a myosin II network in the *Drosophila* follicle epithelium. Representative examples of dynamic myosin II (MRLC::GFP) behavior (**A** and **B**) at the local cellular scale and (**C**–**F**) at the tissue scale for control and *fat2* mutant *Drosophila* egg chambers. See **Table of Materials** for detailed information on used genotypes. Note that MRLC::GFP signals (green) move perpendicular to the anterior-posterior (AP) axis of control egg chambers. This polarity is lost in *fat2* mutant egg chambers and leads to anisotropic myosin II pulses/oscillations[12]. Upon manual analysis of small MRLC::GFP signals (~300 µm) and the quantification of their angular directional movement as described in the protocol, symmetry breaking of MRLC::GFP signals can be observed with a preference against the direction of the epithelial rotation in an analyzed egg chamber[12] (**Figure 4**). Corresponding movies are: panel **A** = **Movie 1**, panel **B** = **Movie 2**, panel **C** = **Movie 3**, panel **D** = **Movie 4**, panel **E** = **Movie 5**, and panel **F** = **Movie 6**. Cell outlines are shown in magenta. The anterior side is on the left. The scale bars = 5 µm (in panels **A** and **B**) and 50 µm (in panels **C**–**F**).

Table 1: Recommended imaging parameters.

Movie 1: Representative dynamic behavior of myosin II (MRLC::GFP) at the cellular scale in a control *Drosophila* egg chamber. Note that MRLC::GFP (green) prefers to move perpendicular to the AP axis of the egg chambers. Cell outlines are in magenta. Basal view. The anterior side is on the left. The scale bar = 5 µm.

Movie 2: Representative dynamic behavior of myosin II (MRLC::GFP) **at the cellular scale in a *fat2* mutant *Drosophila* egg chamber.** Note that MRLC::GFP pulses (green) and is no longer planar aligned perpendicular to the AP axis of static egg chambers. Cell outlines are in magenta. Basal view. The anterior side is on the left. The scale bar = 5 µm.

Movie 3: Representative dynamic behavior of basal myosin II (MRLC::GFP) at the tissue scale in a control *Drosophila* egg chamber. Note that only a thin basal (outer) MRLC::GFP layer is extracted from almost half of the follicle epithelium of an egg chamber. MRLC::GFP is in green and cell outlines are in magenta. Notice the difference in the level of detail of the obtained MRLC::GFP signal behavior here (representing the tissue scale) as compared to **Movie 1** (representing the cellular scale). The anterior side is on the left. The scale bar = 50 µm.

Movie 4: Representative dynamic behavior of basal myosin II (MRLC::GFP) at the tissue scale in a *fat2* mutant *Drosophila* egg chamber. Note that MRLC::GFP (green) pulses strongly at the basal side of almost half of the follicle epithelium of a *fat2* mutant egg chamber and fails to generate the synchronized force required to promote epithelial rotation. Cell outlines are in magenta. The anterior side is on the left. The scale bar = 50 µm.

Movie 5: Representative dynamic behavior of apical myosin II (MRLC::GFP) at the tissue scale in a control *Drosophila* egg chamber. Only a thin MRLC::GFP layer is extracted from the apical (inner) side of almost half of the follicle epithelium of an egg chamber. Note that MRLC::GFP (in green) shows different dynamic behavior here at the apical side as compared to the basal side of the follicle epithelium (as shown in **Movie 3**). Cell outlines are in magenta. The anterior side is on the left. The scale bar = 50 µm.

Movie 6: Representative dynamic behavior of apical myosin II (MRLC::GFP) at the tissue scale in a *fat2* mutant *Drosophila* egg chamber. Altered dynamic behavior of MRLC::GFP (green) extracted from a thin apical region of almost half of the follicle epithelium of a static *fat2* mutant egg chamber. Cell outlines are in magenta. The anterior side is on the left. The scale bar = 50 µm.

DISCUSSION:

Critical steps and troubleshooting for the dissection and culturing of egg chambers

If too many flies are placed into a small vial, the fly food can turn muddy after 2–3 days due to extensive amounts of feeding larvae and adult flies getting trapped in the fly food. In such a case, flip the rest of these flies into a new vial with fresh food and downsize their number. In particular, exclude females that were stuck in the food.

The Schneider mix (SM) should be prepared in advance and can be stored at 4 °C for ~14 days. Be aware that an older mix may contain crystals that can damage the surface of egg chambers. Always mix the SM with freshly added insulin and allow it sufficient time to reach room temperature. This protects egg chambers against cold shock, which can have a negative impact on the growth of microtubules and the planar alignment of the cytoskeleton (as seen in the developing *Drosophila* wing[19]).

Egg chambers and selected ovarioles are very fragile and, due to their small size, may float in the SMI (SM with insulin). It is recommended to let them spontaneously sink in the SMI. They also often stick to the dissection forceps/cactus tool. In such a case, let them release themselves from

dissection devices by gently moving them in the SMI. Avoid squeezing and touching them directly at all times. If required, exclude these egg chambers/ovarioles from the further protocol.

As a dissection stereoscope is not reliable for the identification of damaged egg chambers/ovarioles, egg chambers/ovarioles should be checked using a CellMask or FM 4-64 dye under a confocal/spinning disk microscope. Damaged egg chambers show extreme coloring as compared to an undamaged egg chamber tissue background. Never acquire a TLM with strong dye patches.

Critical steps and troubleshooting for the in vitro live imaging of egg chambers

If freely placed egg chambers in the SMI still move, check again under the confocal microscope to see whether there are still any overlooked remnants of muscle sheet and debris floating in the SMI. Remove them and try again. Of the egg chambers, 90% should be stable and immobile during subsequent imaging.

To make sure that a selected egg chamber/ovariole is stable, use high-speed imaging (6 s intervals) for 1 min. Unstable egg chambers would move by this point. However, it is recommended to watch the whole time-lapse recording to be able to gently correct a potential unexpected movement of the imaged egg chamber. This can be done by moving the microscopic stage/table, which holds the Petri dish with the cultured egg chambers/ovarioles, to the original position so that the egg chamber of the interest is again in the imaged, focused window.

Stop imaging if a sudden and unexpected movement of the imaged egg chamber appears. Check the cushioning of the microscope table, and avoid walking around near the microscope during the acquisition time as this may result in the disruption of the image acquisition due to the vibrations caused.

If the cell membrane dye is not visible after half an hour and the used laser line is correct, add more of the dye and increase its concentration for the next acquisition.

If the actomyosin signals appear to be blurred, check the NA of the objective used. An NA lower than 1.3 will decrease the imaging quality. Additionally, make sure that used immersion oil has been applied correctly to the water 63x objective. Add or replace it if necessary.

If the egg chambers shrink and the observed cell membranes deform, check whether the lid is properly closed. If the lid is missing or not properly closed, the egg chambers can dry out due to SMI evaporation over the acquisition time.

If the rotation of the egg chambers slows down or stops, decrease the laser power. If a hole appears in the egg chamber, it has been burned by the laser. Decrease the laser power.

If the actomyosin signals bleach after 2 min during TLM acquisition, decrease the laser power and increase the signal amplification in the microscope software.

Once a TLM of the follicle epithelium of one egg chamber has been acquired, it is recommended to avoid imaging again in the same tissue region of this egg chamber. However, as egg chambers rotate around their AP axis, it is possible to repeat the acquisition of a TLM using this undamaged egg chamber after circa 30 min. While imaging the follicle epithelium in one egg chamber, other egg chambers will not be bleached/damaged even if they are located in the same ovariole or in another ovariole in the SMI.

If all these requirements are met, the percentage of successfully imaged egg chambers should be circa 90%–100% for stages 6–8, circa 50%–60% for stages 3–5, and circa 20%–30% for stages 1–2. Failure is mainly due to the movement of egg chambers or damage to them during their dissection/manipulation.

Critical steps and troubleshooting for data processing

During mask generation using the provided script in Fiji, it can happen that there are a lot of generated cell outlines that do not reflect the actual cell membranes in a TLM. This is often caused by high background noise, especially when dyes to stain cell membranes are used. In such a case, to avoid the tedious correction of undesired cell outlines in the generated mask, run the segmentation again and set the parameters to the best fit. This can be done by adjusting the **Estimated noise tolerance** parameter.

When loading one of the ParticleStack.tif files containing cell outlines into Surface manager, the loading time scales linearly with the number of cell outlines and can take several minutes. If the loading is disrupted or incorrect, repeat it. Make sure that the uploading window is in focus and no other program is being used.

Sometimes a cell outline needs to be corrected in some frames; in such a case, use the **Brush** button. Draw the correct cell outline in one particular frame by dragging the mouse around the cell membrane. Move to a different frame of the TLM, and then, return to the time frame with the correction: the incorrect cell outline should now be replaced. Then, go again to the next frame to correct that one. The **Brush** tool will now switch to erase mode. If necessary, correct the outlines in the next time frames by pushing the existing cell outline and then pressing the **+Add** button to create a new cell outline. Delete the old S number from the Surface manager window and rename the new cell outline by pressing the **Rename** button if required.

If an entire important cell is missing throughout a TLM, create a new mask outline by pressing **Unselect** > **Polygon**. Create a new cell outline in the first frame of the TLM by clicking along the cell membrane. Then, go to the last frame of the TLM and do the same for the selected cell. By running the movie in time, the cell outlines will be interpolated. Correct with the **Brush** tool if necessary. Once finished, press the **+Add** button and rename the cell outline by pressing the **Rename** button. The more points/clicks to create a new cell outline, the better interpolation works.

Besides epithelial rotation, TLMs are sometimes affected by unwanted movement. Therefore, it is recommended to correct for such tissue drift in these TLMs. By doing so, TLMs are also

corrected for the epithelial rotation of egg chambers, and cell membranes become rigid. This makes the manual analysis of actomyosin signals easier. However, such an approach does not allow scientists to distinguish whether the observed actomyosin signals move or are static relative to the cell membrane. If a distinction between static and active movements of actomyosin signals is the goal of such an analysis, no tissue drift correction should be applied. We found that the majority of actomyosin signals actively move relative to the cell membrane and only a minor portion of them appear static.

Critical steps and troubleshooting for the analysis of subcellular actomyosin signals

If the statistics generated contain outliers, ensure that any extremely low or high values with respect to the whole data set are truly reflecting the behavior in the cell and are not artifacts. This can be done by identifying the cell containing the outliers and checking for cell outline quality at the time when the low/high value was measured. Often, such extreme values result from defective cell outlines that incorrectly measure part of another cell. This may be particularly apparent when large blobs interfere with the cell outline of a neighboring cell. In such a case, it is crucial to correct the cell outlines and repeat the measurement.

To make the quantification easier, analyze the signals in one particular cell surface and continue one by one until all cells are analyzed in a submovie. The results of manual analysis of subcellular actomyosin signals may not show symmetry breaking in one cell and one particular egg chamber. We experienced that the actomyosin does not clearly break symmetry relative to the tissue movement in <10% of the rotating egg chambers (stages 6–8). This percentage is increased around stage 4[12]. We also found that there is no difference whether 5 min or 10 min are analyzed in the identification of the preferred direction of actomyosin signal movement in an egg chamber[12].

Critical steps and troubleshooting for the selective extraction of actomyosin signals from curved tissues

The wrong size of blobs (identified signals) will result in no blobs and the program will freeze in the next step. In this case, force-quit Fiji and newly restart the plugin **Ellipsoid Surface Projection**. Do the same when the program does not react after pressing **Compute**. This is often an indication that unsuitable parameters have been chosen.

If there are too many blobs and/if they are concentrated toward one side of the analyzed egg chamber, this may impact the designed ellipsoid and not provide the correct fit for the egg chamber. Go back to the blob identification and try to arrange their size in the combination with the x-, y-, and z-axis selection of the egg chamber. A failure to generate a good ellipsoid fit also often occurs when unsuitable cut-off distance settings are used.

The projections obtained may sometimes look misfocused, or perhaps the obtained z plane actually moves between cell layers near the surface of the egg chamber. This is usually a sign of a poorly fitting ellipsoid where one region of the ellipsoid is set too far from the egg chamber. Try to fit the ellipsoid so that it maintains the same distance from the egg chamber circumference.

Limitations of the method and novel approaches

This free culturing of egg chambers omits the subtle flattening of an egg chamber's surface. To this end, this method has its advantages and disadvantages. When using confocal microscopy, it provides users with high-resolution and high-speed imaging that reliably uncovers actomyosin behavior in cells at the circumference of egg chambers for a short period of time (5–10 min). However, by doing so, it limits the size of a single plane that can be imaged with confocal microscopy over time. In general, it is possible to image up to ~15 cells per egg chamber at stages 6–8 but only about two cells in egg chambers at stages 1–5. Therefore, we have defined this method here as being suitable only for the local cellular scale.

To overcome these size limits that are caused by the limited size of a single confocal plane, we have developed an alternative Fiji-based approach called Ellipsoid Surface Projection, for use at the tissue scale. This combines spinning disc microscopy with a semi-interactive surface extraction of egg chambers at the tissue scale. In this way, actomyosin signals can be obtained at the same time for more than 50–100 cells from one analyzed egg chamber. It is important to note that this approach generates very large datasets of the acquired data (~giga bytes), has a lower resolution (it uses a 40x vs. a 63x objective), and also provides a slower imaging speed (it takes ~60 s to scan through half of the tissue of an egg chamber [stages 6–8] and, as such, it is 10x slower than the limited confocal plane method).

Compared to other existing software for extracting layered projections from parametrized surfaces, the plugin developed for this protocol is focused on ease-of-use and interactive visual feedback at every step of the process. Other tools, such as the MATLAB-based ImSaNE[20], used by Chen et al.[21], focus on handling a wide variety of parametrized and nonparametrized surface models and various projection methods. For example, ImSaNE requires data to be preprocessed and aligned in a particular way and partially requires external tools in intermediate steps. In contrast, the plugin presented here handles any 3D/4D/5D image (sequence) that can be opened in Fiji without external preprocessing. While ImSaNE is highly configurable (programmable) by editing MATLAB scripts, we provide a minimal set of options in one workflow that is tailored to the specific problem discussed here. Each step of the interactive workflow provides results that can be immediately visually inspected and adjusted if necessary.

The decision as to which of these imaging approaches, the local cellular or tissue scale, is the best for a particular experiment, depends purely on the scientific question to be answered (i.e., higher vs. lower resolution; short vs. long acquisition time). A good compromise between these two scales could be to combine both approaches, thus gaining the imaging of actomyosin signals at the semi-tissue scale (i.e., 20–30 cells). This requires a spinning disc microscope, a water 63x lens with NA ≥1.3, and established z-stack settings for 20–30 cells of an egg chamber. This much shallower z-stack (in contrast to the z-stack required for the acquisition of one half of an egg chamber) allows faster scanning of under 60 s. The time gained here can be used either for repeated z-stack acquisition to speed up the imaging or for a sample recovery (time interleaves) between individual z-stacks. With the latter, a longer acquisition time (>30 min) of TLMs of rotating egg chambers can be achieved. This semi-tissue approach guarantees a sufficient

resolution for actomyosin signals and the imaging of more cells at the same time over longer time periods.

Future applications and vision

Both described methods (at the local cellular and tissue scale) provide a simple and low-cost approach (excluding microscope devices) with limited side effects on the actomyosin network and can be implemented and easily adopted for other dissected animal tissues. The only prerequisite here is an existing culturing protocol in a Petri dish for a curved tissue of interest, available transgenes, and markers or labeling methods.

In combination with light-sheet fluorescence microscopy[22], it is also possible to image actomyosin machinery in toto (i.e., image the complete outer circumferential surface of *Drosophila* egg chambers simultaneously and then subsequently unfold using the plugin **Ellipsoid Surface Extraction**). However, there are a few limitations that need to be resolved in terms of suitability for high-speed imaging of actomyosin signals, namely 1) the embedding of egg chambers in a low-point melting agarose or their sticking to a capillary during imaging; ii) a low numerical aperture of used water lenses that do not provide sufficient actomyosin signal resolution; iii) the additional time needed to acquire several angles of egg chambers, which prevents high-speed imaging.

To this end, it is foreseeable that, with the refinement of microscope parameters such as the speed to scan through the epithelial tissue and the improvement of used microscopic lenses, the detailed analysis of actomyosin machinery will, in the future, enable promising high-resolution results to be obtained at the tissue and in toto scale over long time periods.

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The authors have nothing to disclose.

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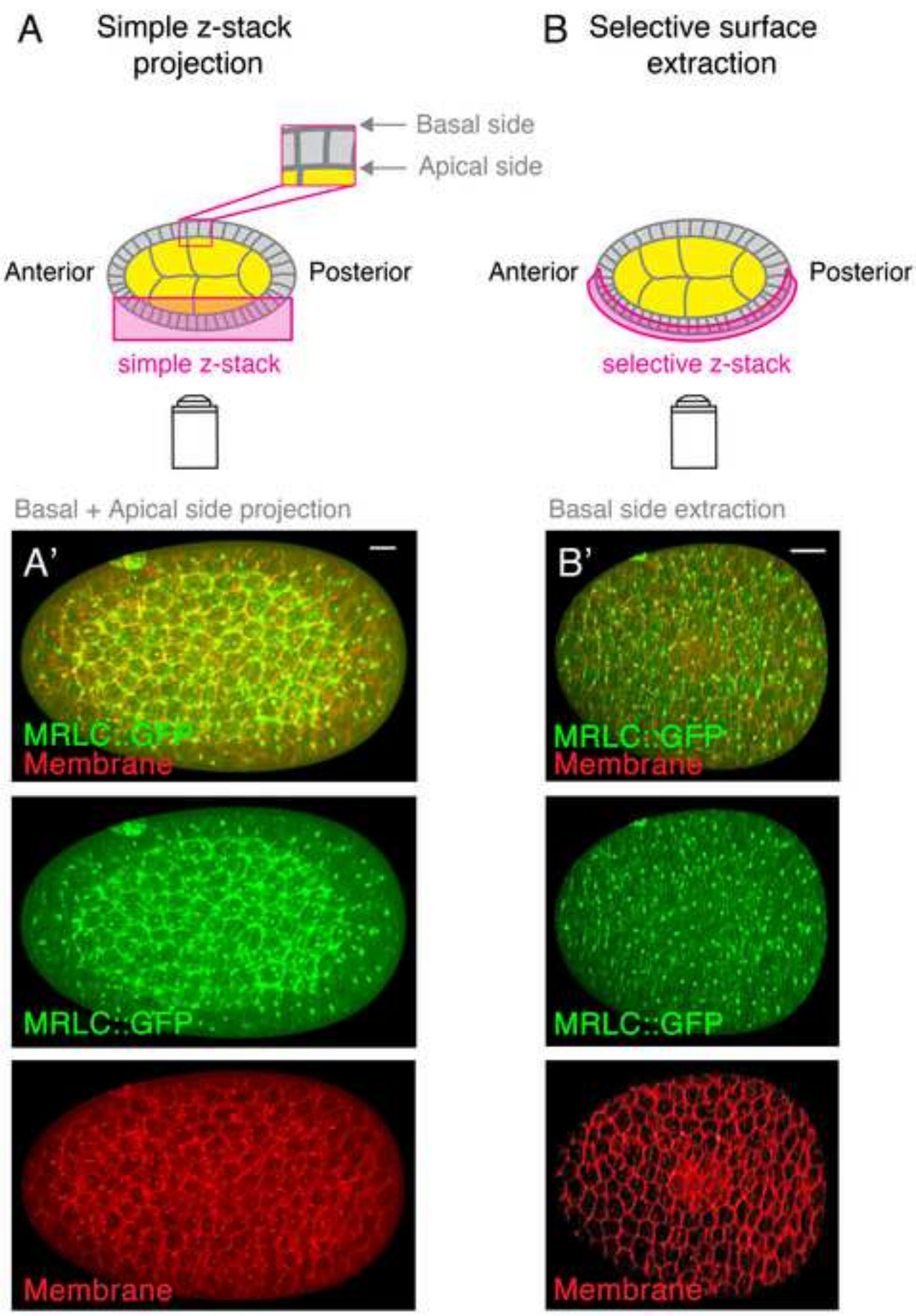
Figure 1



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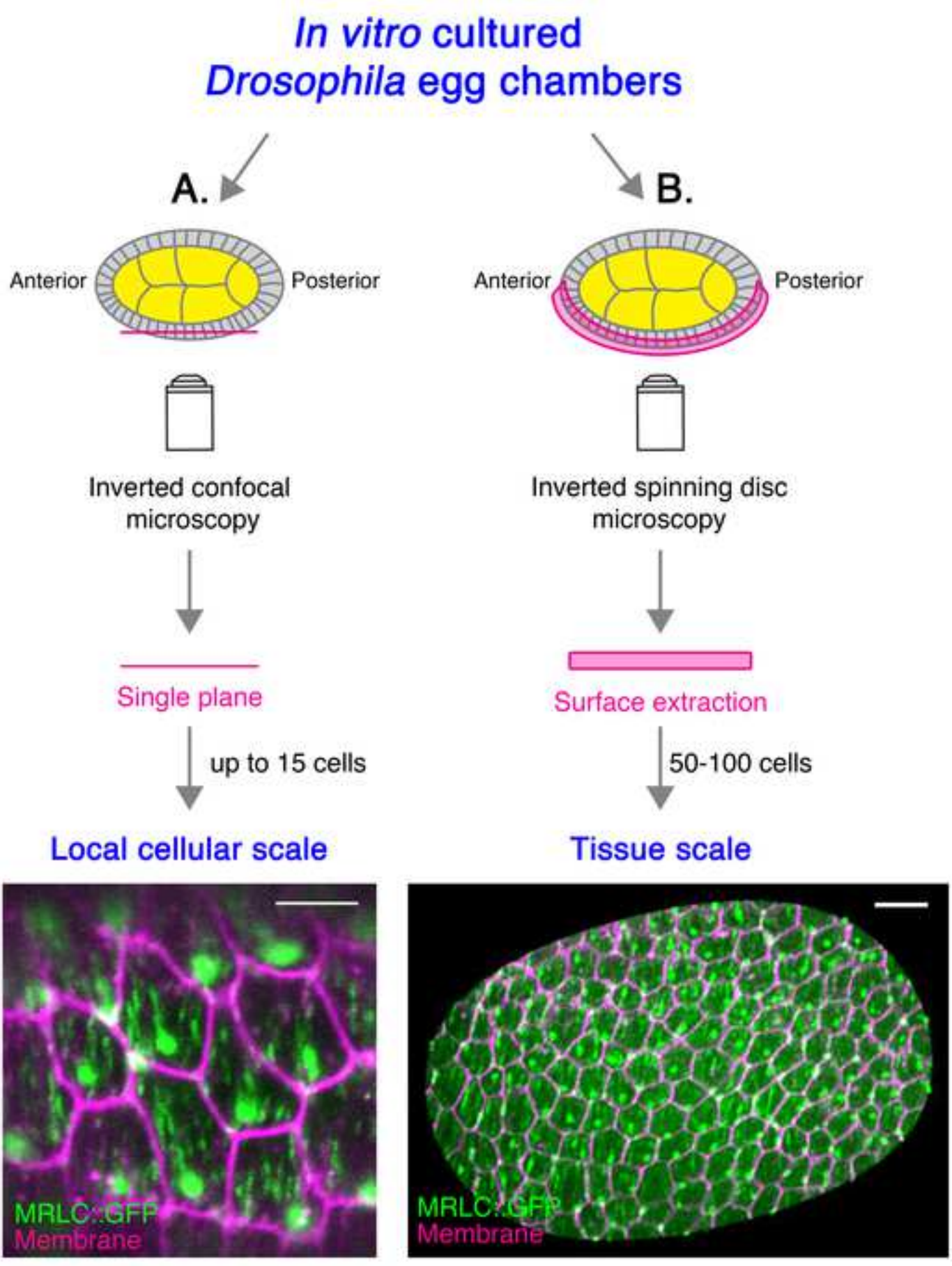

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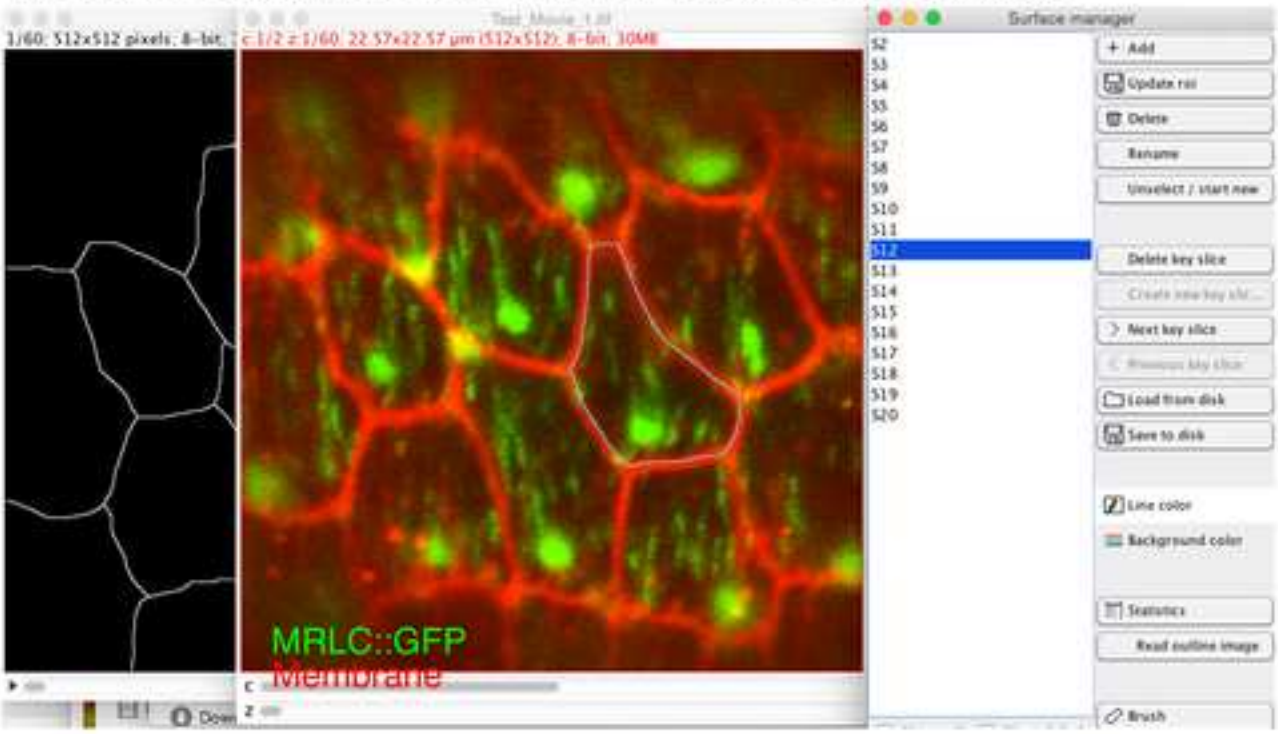
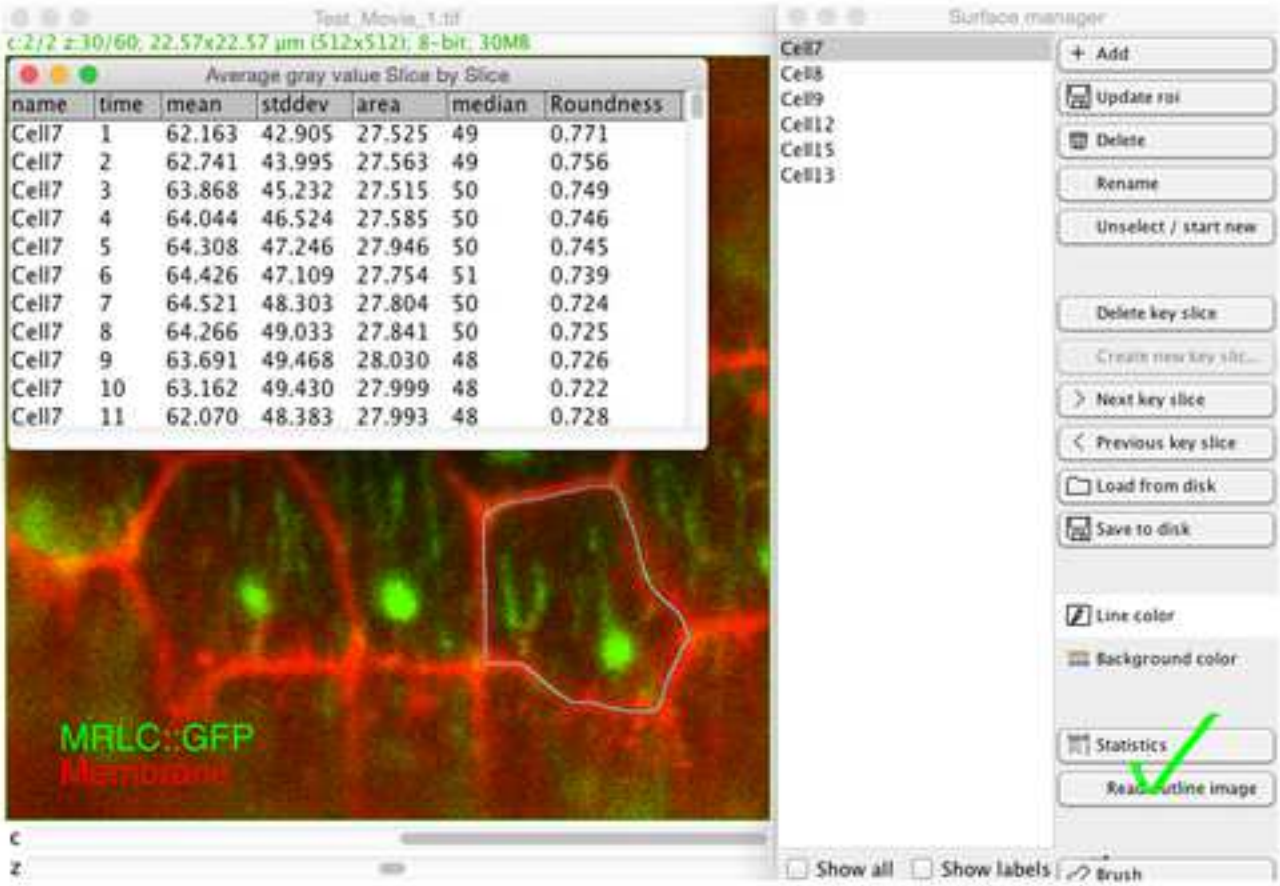


B. Example statistics for selected cells in Surface manager



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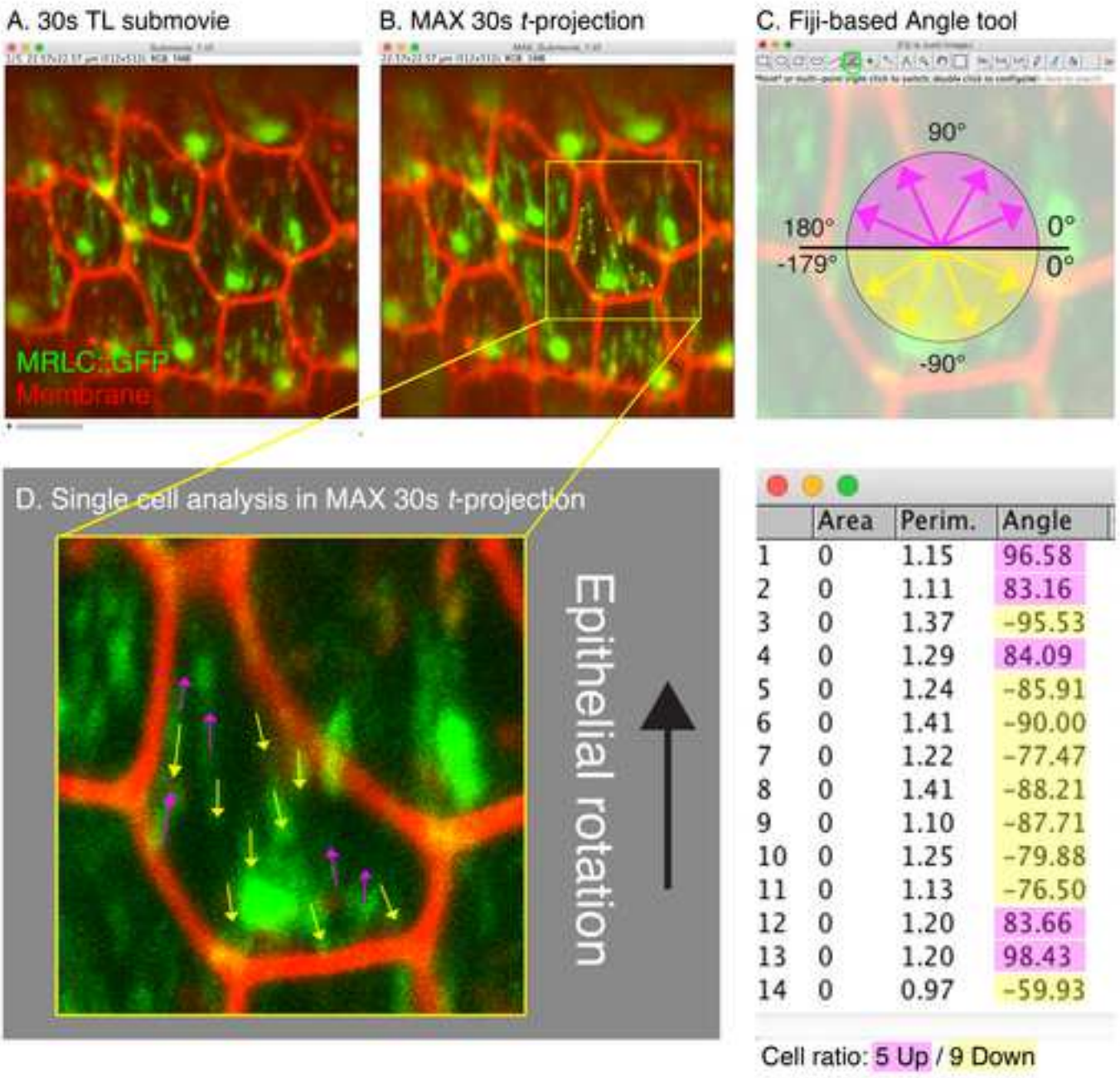

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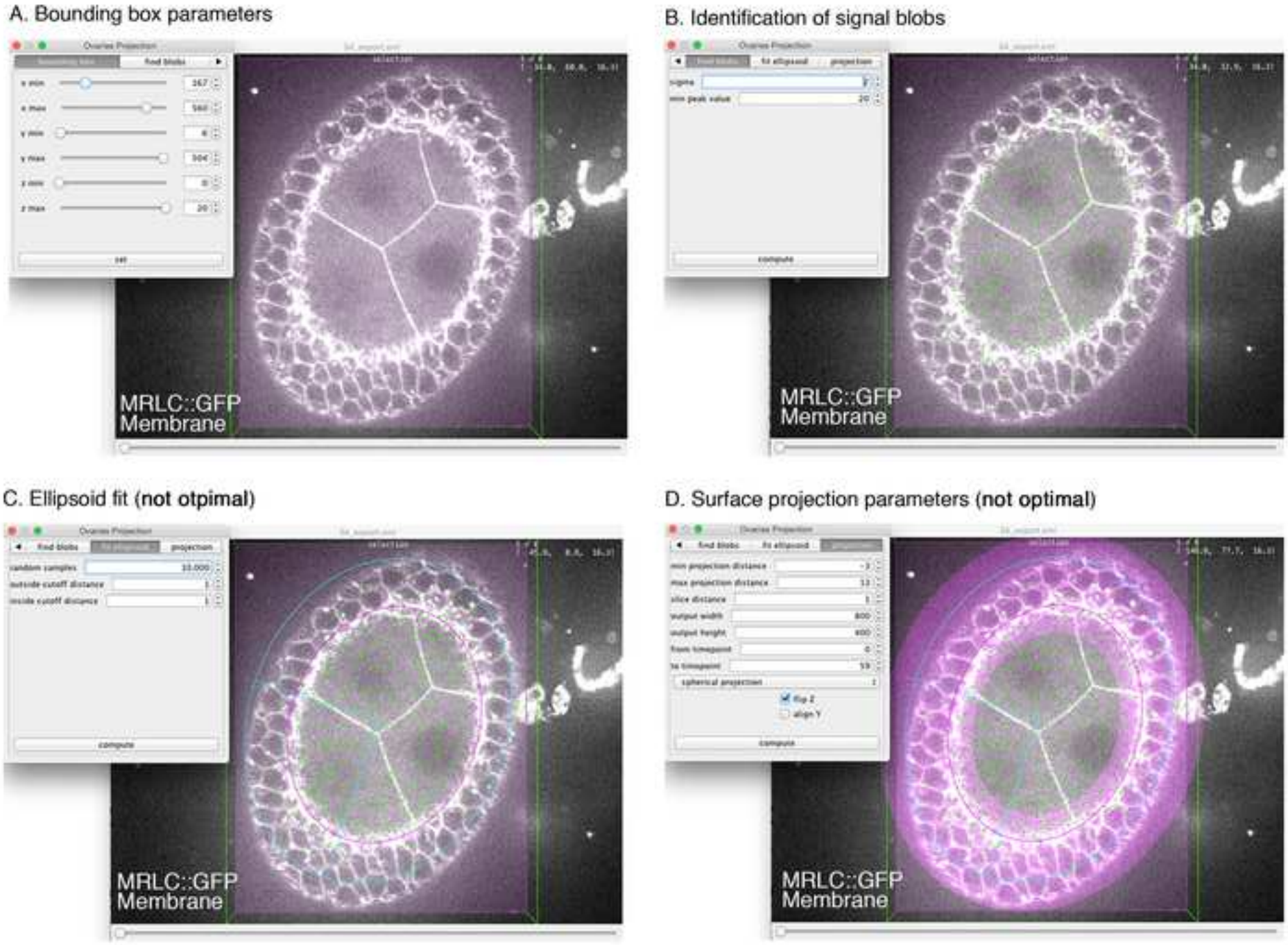


Figure 6

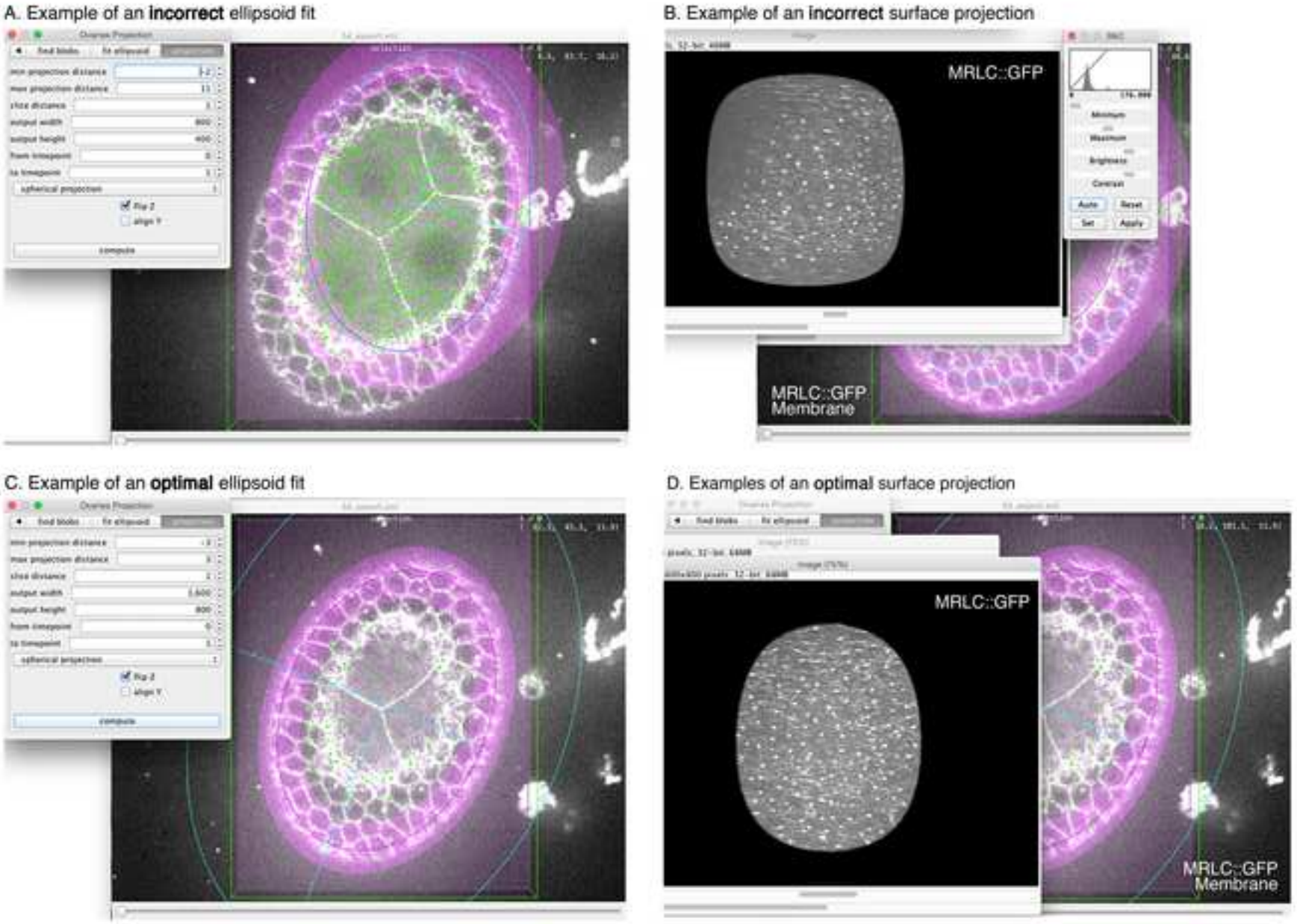
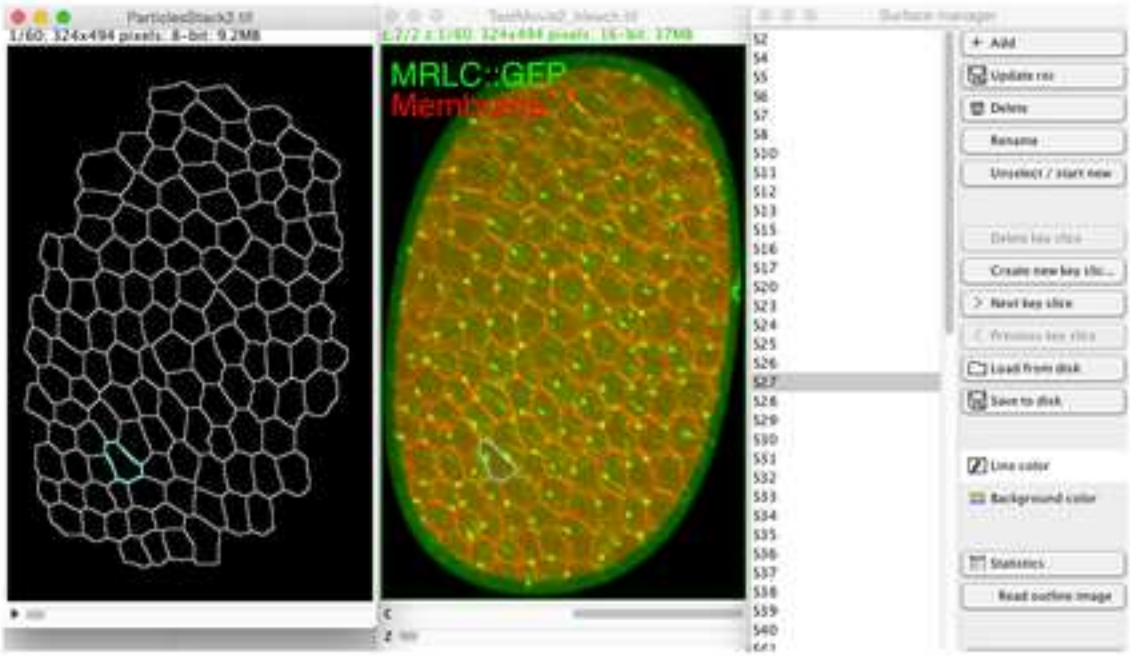
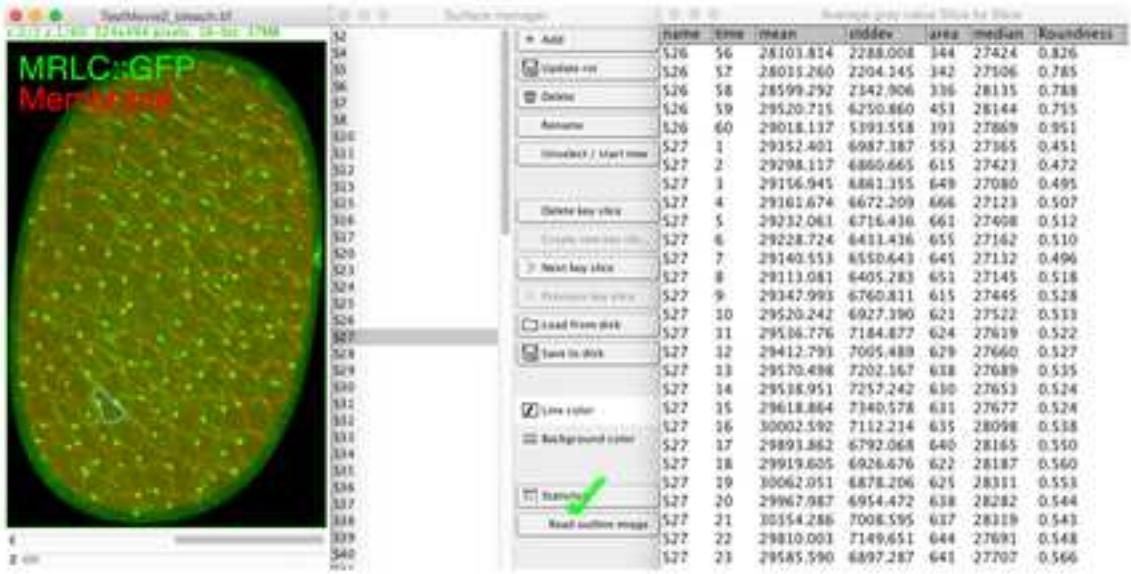


Figure 7

A. Example of a TLM with a loaded particle stack in Surface manager



B. Example statistics for selected cells in Surface manager



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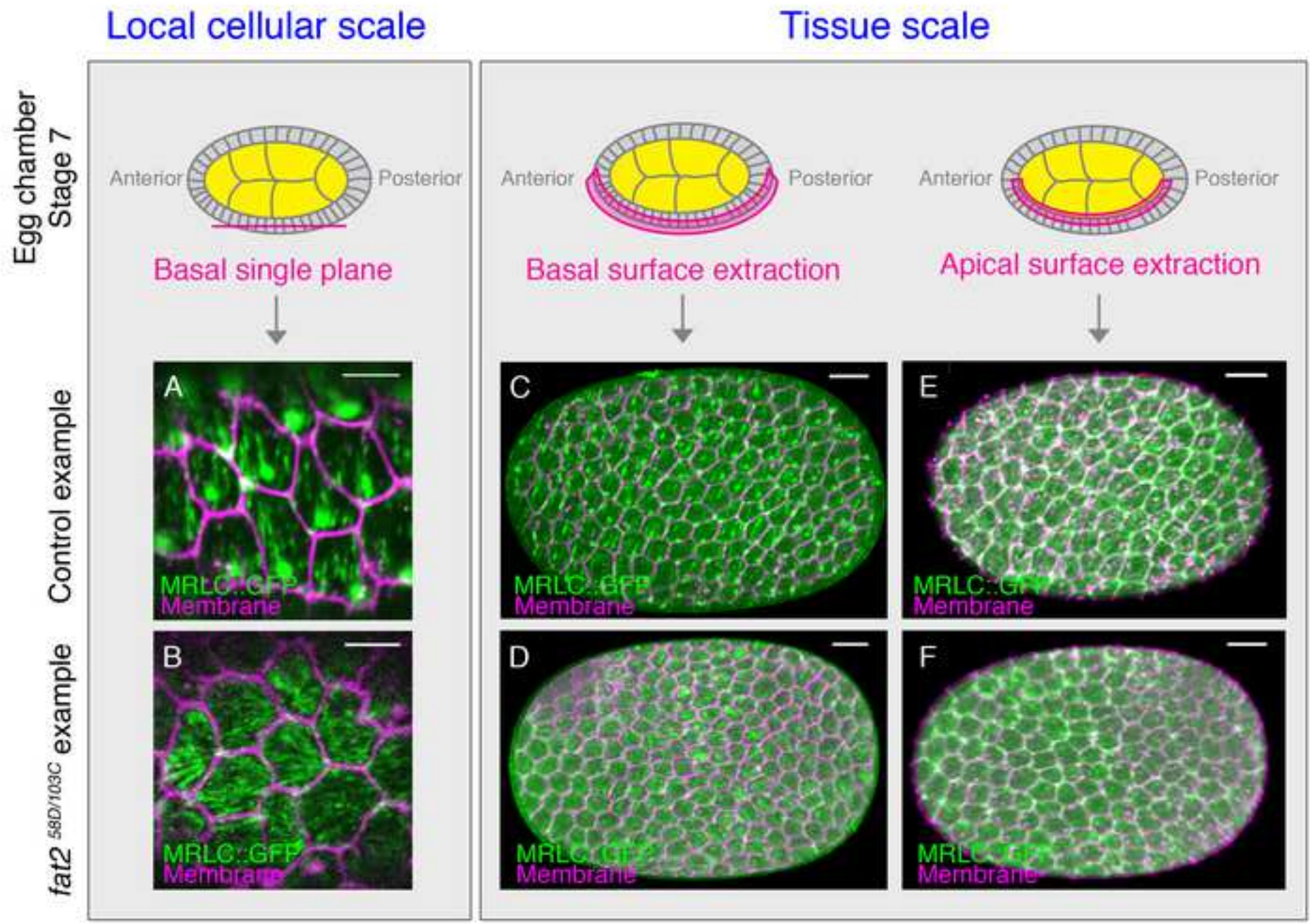

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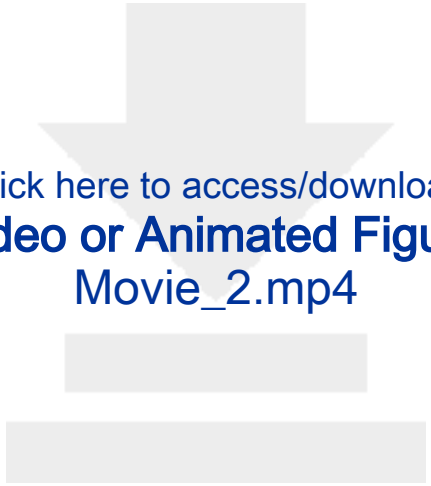

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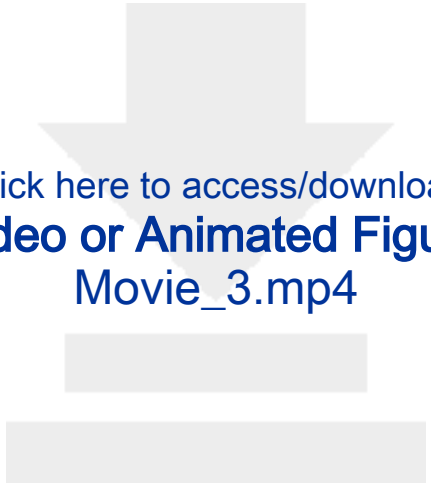

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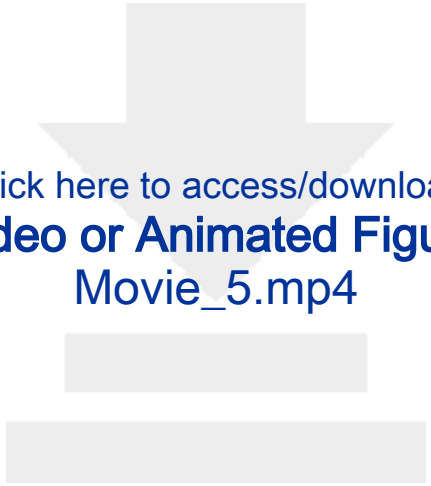

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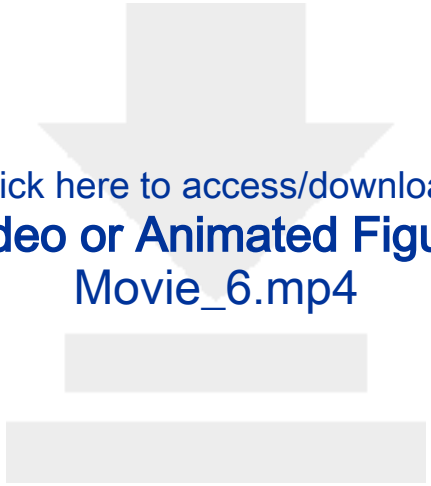

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Table

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ıded parameters for short-time high-speed imaging at the local cellular scale:

Tissue of interest freely placed in the culturing medium (i.e. no cover slips, flexible blankets, etc.)
63x water objective with numerical aperture >= 1.3
Inverted confocal microscope
Single plane
6-12 s time intervals
5-10 mins TLMs
Data storage requirement per TLM up to 100MB

ıded parameters for long-time low-speed imaging at the tissue scale:

Tissue of interest freely placed in the culturing medium (i.e. no cover slips, flexible blankets, etc.)
40x water objectives and numerical aperture >= 1.3
Inverted spinning disc microscope
z-stacks to acquire ca. half of an egg chamber
60 s time intervals
At least 30 mins TLMs
Data storage requirement ca. 1GB

Name of Material/ Equipment	Company	Catalog Number
Schneider Medium	Gibco	21720-024
Penicillin/Streptomycin	Gibco	15140-122
Fetal Bovine Serum	Sigma	F135
Insulin Solution Human	Sigma	I9278
50 ml Falcon tubes	Eppendorf	
Millex-GV filter	Millipore	SLGV033NS
Glass Bottom Microwell Dishes	MatTek Corporation	P35G-1.5-14-C
Forceps	A. Dumont & Fils	
CellMask Deep Red (cell membrane dye)	Invitrogen	C10046
FM4-64 (cell membrane dye)	ThermoFisher/Invitrogen	T13320
Depression dissection slide	Fisher Scientific	12-560B
Microscopic equipment		
Steromicroscope	e.g. Zeiss	
Inverted confocal microscope	e.g. Zeiss/Olympus	
Spinning disc microscope	e.g. Zeiss/Andor	
Microscopic glass/cover glass	e.g. Thermo Scientific/Menzel Glas	
Cactus tool		
Wironit needle holder	Hammacher	9160020
Cactus spine	Echinocactus grusonii/barrel cactus	

Drosophila stocks used in the manuscript

AX^{3} /AX^{3} ; sqh-MRLC::GFP/sqh-MRLC::GFP

AX^{3} /AX^{3} ; sqh-MRLC::GFP/sqh-MRLC::GFP; fat2 ^{58D} /fat ^{103C}

Comments/Description

[+]-Glutamine
[+] 10.000 Units/ml Penicillin [+] 10.000 µg/ml Streptomycin
Heat inactivated

sterile
33 mm
35 mm Petri dish, 14 mm Microwell, No. 1.5 cover glass
#55

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For detail see Rauzi et al.: Planar polarized actomyosin contractile flows control epithelial junction remodeling. Nature 2010, Vol. 468, pg. 1
For detail see Viktorinova et al.: Epithelial rotation is preceded by planar symmetry breaking of actomyosin and protects epithelial tissue fro

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Author(s): Ivana Viktorinová, Robert Haase, Tobias Pietzsch, Ian Henry and Pavel Tomancak

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Alisha DSouza
Senior Review Editor
JoVE
Cambridge, MA
USA

18th December 2018

Dear Alisha,

Please find attached our revised manuscript Nr. 58587 titled 'Analysis of actomyosin dynamics at local cellular and tissue scales in cultured *Drosophila* egg chambers' that we resubmit to Journal of Visualized Experiments (JoVE) after the first round of revision.

We would like to thank you, your colleagues and all three reviewers for your and their thorough comments and suggestions. Upon revising our manuscript according to the reviewers' wishes, we resulted with a protocol that far exceeded the allowed length. This prompted us to restructure the protocol section and place several instructions (dissection and imaging of egg chambers) into the supplement. Therefore, the main part of this revised protocol focuses on actomyosin analysis at the local and tissue scale.

We provide only a few links to publicly accessible git-lab repository (https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics) and other external sources. However, this can be re-arranged according to your policies. We hope that through this restructuring, our protocol is now easy to follow and user-friendly to a broad spectrum of readers, even novices.

We also provide high resolution figures. We apologize for the previous issues, which must have happened when converting our files into the formats required by JoVE. Please, let us know in case this remains an issue.

Please, also find attached our detailed responses to the reviewers below.

We thank you again for considering our manuscript for publication and are looking forward to hearing from you.

Yours sincerely,

Ivana Viktorinová and co-authors

CC: rhaase@mpi-cbg.de, pietzsch@mpi-cbg.de, henry@mpi-cbg.de, tomancak@mpi-cbg.de

Dear Dr. Viktorinova,

Your manuscript, JoVE58587 Analysis of actomyosin dynamics at cellular and tissue scales in cultured Drosophila egg chambers, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

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Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) your protocol steps.** Furthermore, there should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples of missing details:
1) 1.1: Is the fly food placed in a vial? How much per vial? We now provide this info.
2) 2.2: Remove particles by pouring through the filter? We now state to remove particles by using the filter.
3) 2.8: Use the word “anesthetized” in place of “sleeping” We now use the word ‘anesthetize’, thank you.
4) 3.2.1.2: Split channels how? We now describe how to do it.
5) 3.2.1.2: Merge how? We now also added this information.
6) 3.2.2.2: Run how? We now provide this info.
7) Line 234: how? We now describe this.
8) 3.5: Is this done in FIJI? Yes, all steps are Fiji-based as we state now so.
9) 3.5.3: how? We now describe this in the note.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE’s instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.
ok
• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE’s instructions for authors for more clarification. Remember that the

non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
3) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
4) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
5) Notes cannot be filmed and should be excluded from highlighting.
6) Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.

• **Discussion:** Please avoid using a number list in this section.
ok
• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Millex®, MatTek, CellMask, MatLab, all items listed in the supplementary materials list following the protocol.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

• **Table of Materials:** Please remove the Supplementary material section from the manuscript and list all materials in the table of materials. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file.

• Please define all abbreviations at first use.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:
An important step early in Drosophila oogenesis is rotation of follicles around their A/P axis. Breaking of Myosin II symmetry leads to directional actomysin activity that generates the forces necessary to drive rotation. Understanding the action of these forces can provide insight into cellular movement in general. Ideally, in vivo analysis at the level of individual cells and/or at a tissue level is required to understand the role actomyosin. The manuscript provides alternate procedures to prepare samples for live imaging in a way that circumvents distortion of cell shape that occur with previously published live imaging methods. Beginning with dissection of ovaries, this procedure describes the isolation of early stage eggs for analysis and employs a coverslip-free method to culture egg chambers during imaging to eliminate potential sources of cellular distortion while minimizing sample movement, thereby providing a more accurate depiction of actin dynamics. It then provides a step-by-step guide in the use of image analysis techniques for the analysis of actomyosin dynamics in time lapse images for both single plane and thick tissue samples. This manuscript is highly suitable for publication in JoVE as both the dissection and image analysis steps will benefit from the video documentation features. However, the manuscript suffers from a lack of clarity about both the rationale and the experimental protocol. Thus, extensive changes are required. Listed below are specific suggestions to assist the authors.

Lack of clarity:
1) The issue of tissue curvature and the need to correct for this to analyze the image data is not clearly explained. This aspect need to be discussed in the introduction and discussion.

Thank you for this point. We now explain why is that important in the text.
2) It would be extremely helpful to provide an example data files for the reader to analyze using the methods (one for 3 and one for method 4).
We agree and now provide example data sets to individual steps.
3) Larger and more detailed figures are needed.
We are sorry that you obtained low resolution data. Our psd files are 600 dpi and generally 3000x5000 pixels, the low resolution must have happen by a conversion of those file. We hope that now we provide high resolution figures that can be easily read.
4) The output data and their purpose are not clear (i.e. 3.3.1 and 3.3.2). An example of how the data is used would help (i.e. comparison of wild-type and mutant).
We hope that in the revised manuscript it is clear.
5) The image adjustment and analyses the settings used are not explained, nor are alternatives discussed.
We now explain.
6) What is actually being done in the methods steps are unclear. A short sentence stating something like: Set up an image export path by…..would dramatically improve the clarity of the methods. This type of change is needed throughout.
We now provide snapshots/screenshots which should help the clarity and detail action guidance.

Specific comments:
1.Introduction
Line 82: change media to medium
Thank you. We have corrected as suggested.
Line 100: In relation to the discussion of pressure in other live imaging mounting techniques, it seems important to consider that the egg chambers are normally under pressure from the muscle sheath and adjacent chambers. Thus, no pressure may be artificial.
We believe that classical glass cover slips provide a different type of pressure than surrounding soft muscle sheet and/or adjacent chambers. In fact, the question is whether egg chambers are really pressed in ovaries. One has also to take into a consideration that glass material is not present in ovaries and may have a certain antistatic properties. Nevertheless, we agree with the reviewer's point that certain pressure might be ok and actually cause no harm to egg chambers. However, it would be necessary to empirically test to prove/disprove that no pressure is artificial in this case.
Line 102: change life to live
Thank you for finding this typo. Now, it is corrected.

2.Protocol

For this section, it might be useful to have a figure that shows what ovaries look like and a schematic of the steps in dissection, particularly ovarioles with and without muscle sheath.
Thank you. We describe it hopefully better in detail.
Line 135: How are you taking the female?
Now specified.
Line 140: Wording that the ovaries and other organs will "fall off" perhaps isn't the best choice of phrasing. It might be better to state that the ovaries, along with other organs will be pulled out of the abdomen.
Arranged as suggested.
Line 142-143: Phrasing. I would not describe ovarioles as fibers, rather they are chains or strings of developing egg chambers. We use now the word string. Additionally, how are the ovaries being moved and how are the ovarioles being dissected?
We now describe, how to pull out one ovariole out of ovaries.
Line 142-156: You need to explain the rationale for why it is necessary to dissect the ovaries in this manner- is it necessary to reduce the risk of damage, etc.? Now we include that by dissection ovarioles can be damaged.
Why not separate the ovarioles in the original drop, then transfer the chosen ovarioles to the MatTek dish? This would reduce/eliminate debris in the MatTek dish and reduce the risk of ovarioles drying out when replacing dirty SMI. We agree that this approach would avoid debrits in the dish, however, to transfer a single ovariole is a risk. It can stick on the forcep and by trying to release it means another danger to damage egg chambers in ovarioles. Therefore, we prefer to let ovarioles to only gently pull out from ovaries and limit their touching.

Line 152: What is meant by remnants released from the female abdomen? Now we removed remnants and specified what we mean. Also, since ovaries are the topic in this paper, it is not necessary to specify female abdomen.
Line 158: Can the dye be added to the SMI prior to adding the ovaries? This would reduce the risk of damaging the egg chambers when mixing the dye. We agree that it is also the way to do it. However, the SMI often needs to be replaced/cleaned from debris and old egg chambers. Our way, therefore, requires to place the dye only once to the SMI. Mixing with a forcep is a gentle step. To make sure that the reader should be careful, we add: 'Avoid contact of ovarioles with the forcep…'
Line 161: Phrasing. Change "You might need to figure out how long and what dilution works the best in your case." To "Optimization may be required for different applications."

Thank you for this suggestion. We have replaced our text with your suggested sentence.

3.Analysis of actomyosin at the cellular scale

Line 175-181: It seems that six to seven ovarioles would be a lot to have in 100ul of SMI, and it thus be difficult to prevent them from contacting each other. We understand your concern, but based on our experience this number of ovarioles is the perfect number as it is still possible to nicely spread ovarioles in the dish and at the same time it provides enough material for imaging session of 2 h without a need to dissect new ovarioles. The dish has relatively wide well for a good spreading of ovarioles and the bleaching of unimaged ovarioles is close to zero.
Also, the cactus tool is not in the reagent list. We apologize for this. We have add the cactus tool to our reagent list.
Line 184-185: Is a Z-stack being collected? If so, specify. If this method does not require a z-stack, then be sure to specify as such. It is unclear if a single plane or a z-stack are being acquired. If it is a single slice, is the use of a confocal necessary? Additionally, given that all the image analyses assume 63x, it seems like you should indicate what objective to use and why. Thank you for this point. We now specify why to use 63x and provide detailed instructions. To acquire actomyosin signal behaviour, we obtained the best results by using the confocal microscope in terms of signal resolution.
Line 187. What "selected cells" are us unclear? Since the protocol is about follicle cell imaging, a more detailed description is needed here. What cells should be focused on and in what plane? We now explain in detail to focus on the most central part of an egg chamber and the basal (outside) side of follicle cells.
Line 191-192: Change "avoid a negative impact of floor resonation…." to "reduce vibration that could affect imaging" Thank you, we have replaced our text with your suggested sentence.
Line 199-200: These formats are specific to specific microscopes. However, many of those who use this protocol may have instruments from other manufacturers. It would be better to state to make sure the TLMs are saved in the confocal manufacture software format and why this is important. We now state that any formats that can be opened in Fiji are possible to use.

3.2 Data processing

Overall, more detail/explanation is needed. A flowchart of the process would be useful in providing an overview of the analyses and the purpose of each step or data. I think this section should begin with ensuring that the user has first installed Fiji and the required plugins, if they have not already done so. It would also be helpful to specify at the beginning what plugins are necessary to complete this procedure so the user of this protocol can easily follow along with an example. Furthermore, a sample image and script would also be beneficial and allow the user to work through the protocol. Finally, figures for each of these steps would be extremely helpful, particularly easy to read images/screenshots highlighting options required for successful image processing.
We now reworked our manuscript in the way that the dissection and imaging part is in the Supplement. We therefore focues on the data processing what was the main aim of this publication. We provide improved instruction accompanied with test files and screenshots in figures.

Line 204: why is bleaching correction required? Setting choice is unclear.
Acquired TLMs suffer from bleaching due to the high-speed imaging and therefore, bleaching correction is highly recommendable to be able to properly see the actomyosin signal later in movie frames during the manual analysis. Setting choice of 0.0 is the standard one. No difference was observed when the value was changed to e.g.10.
Line 206-207: Simple ratio works best if the intensity does not change dramatically. Would histogram matching be a better option for time lapse? You are right. Thank you for this detail. We now provide this info as a note.
Line 211: it is unclear what "TissueCellSegmentMovie-3.ijm" is. Is it a file? Is it a script?
This is a script. As it is not our script, we did not wanted to change its name. We now specify that this file is a script in the text. We provide the link to the original webpage as well as direct link to download it.
Line 212: What is this script, where did it come from, is it specific to a particular image, and does the user have to write this script? If the user must write a script, you will want to address that. How does the user run the script?
As mentioned above, this file is a script developed in other institution (Barcelona by Sebastien Tosi) and you can open this script in Fiji. We now state how to run the script in the text more in detail. Please follow our new instructions.
Line 214: How were these settings chosen and what do they mean?
These settings are the best observed for 63x magnification time-lapse movies and work the best for cell segmentation in stage 6-8 egg chambers.
Line 215: Is this applicable only to a particular brand of 63X objective, or is it universal? Also, if a user wishes to use a 20X or 40X how would they determine the necessary noise tolerance?
It is right that these settings are applicable for 63x objective, we state now that different parameters are required for other objectives. We also state that the cleaner the background, the lower noise tolerance is required.
Line 216-217: Maybe change "clear outlines" to "well-defined outlines". We use now well-defined. How are the cells selected? We state now: well-defined and complete cell outlines.
When was the mask created? Generally, a better description of creating and editing the mask is required (i.e. where is the merge tool).We now provided detail description how to create and edit the mask in the text.
Line 218: Is ParticleStack the filename generated by the segmentation process? If so, it is recommended that

you state what files are generated by the program. We included it into the text.
Line 223: Note that the user must download and install this plugin. It seems like a Fiji set up method is needed. We now start the section of cell segmentation with telling the user to open Fiji and download the script.
Line 234: Describe how to remove unwanted cells. We now describe it.
Line 235: A more detailed description of how to correct the cell outlines and where the "brush" tool is needed. We now added a whole new section how to do it.
Line 238: You assume the reader knows a lot - Where are the tools you are referring to? How is a cell mask created? Where is the "+add"? Added.
Line 246. the phrasing is unclear "the subject to an analysis" We removed this part of the sentence.
Line 252: Method 3.3 needs a short introduction to explain the purpose of the analyses
We have now included brief explanation.
Line 254: It is unclear which file should be opened. Also, why are these cell parameters needed? What can they be used for? We now explain in the introduction and provide instructions in detail.
Line 255: Again, what is the data used for? What is surface manager (where is it in Fiji)? Is it Surface Manager 3D? What is different about the data in 3.3.1 vs 3.3.2? When performing the 'Statistics' function, is it possible to select particular values of interest for analysis? If so, explain how this is done. what is different about the data in 3.3.1 vs 3.3.2? We apologize if our instructions were confusing. We now specify and explain more in detail. We have changed the statistic parameters to relevant ones that are related to our previous paper Viktorinova et al. (2017) PLoS Genetics.
Line 265: Method inconsistencies - you don't always state what program to open the file in.
We now guide the reader hopefully better.
Line 271. Are all the plugins in Fiji generally loaded? If not, you need to add that as a step in the method. Also explaining what each change is in non-Fiji language would be helpful. We now include at the beginning of Data processing section/instruction tutorials how to download and update Fiji to have required plugins.
In general, why is this method important for the later image analysis?
To correct for drift/movement prior to the manual analysis could be probably skipped, but generally, it is easier to detect signal movement relatively to the rigid cell membrane than without the drift correction. Even the short signal movement is then prominent. We included a short explanation by this point in the manuscript.
Line 274: Explain why you use the default values for Gaussian Blur here and if it would be necessary to change this. We tested the MultiStagReg also without Gaussian Blur and it works too. We now put the Guassian Blur and conversion to mask as an optional step.
Line 278-279: What is the purpose of these steps? These steps serve to create the mask based on which the registration/drift correction will be done.
Line 287: it is unclear what step this is an alternative for. We now specify in the text.
Line 291: Method 3.5 An introduction of what the purpose of the analysis is and this data is useful for would be helpful. An example of using the data could increase clarity. We now included a brief explanation of this method.
Line 298: How is the movie divided into submovies? We now explain how to create submovies.
Line 302: What do you mean by signal line? How is a signal line identified? We now specify that a signal line is the trajectory of one actomyosin signal movement over time…
Line 311: Are the same signal lines being analyzed in each submovie? No, we state in the manual that this must be avoided. How many lines are analyzed per cell? How many cells per image? We now state that all visible actomyosin signals should be analysed and it should be aimed for 20-30 signals per one analysed cell. All cells with well-defined outlines should be analysed as we newly state.
Line 312: unclear. We corrected it.
Line 315-317: Is this done in Fiji or exported and analyzed elsewhere? Please explain further. We provide detail instructions.

4. Analysis of actomyosin at the tissue scale

As with the section above, this protocol would benefit by the addition of more explanation of what is required for, and what is accomplished by each step. Again, more figures of dialog boxes, etc. and another example file for the reader to work with would be beneficial.

Line 322: delete e.g. Ok.
Line 331: How big of a Z stack is needed and why. What magnification is needed? Should it be optimal Z-stacks or over/under sampled? What cells are the focus? We now specify to use 40x to acquire tissue scale (half of an egg chamber) that individual planes should sufficiently overlap. As we think that the settings may differ for different spinning discs, we try to avoid too much of a detail.
Line 335-337: This is confusing. May need to clarify point 3.1 to indicate that only a single plane is being collected. We now specify.
Here in the Note, "set up additionally a z-stack" implies that both a single plane and a z-stack are being acquired. We now removed additionally. Is the 60 second acquisition time for your particular settings? It would seem scan speed, step size, number of channels, etc. could all influence this. Yes, we agree. We have corrected it and state that it all depends on the used microscopic settings.
Line 343: change designed to designated We rather deleted this word.
Line 346: Fiji can automatically update upon starting. Is this step absolutely necessary prior to running this protocol? You are right, in this case, it is not required, but we state to install the required plugin.

Line 347: What is the file? What is the purpose of this step? You jump in here with no real explanation. Is there a file open? We corrected this. Thank you for pointing this out.
Line 355: In the note - grammar issues make it confusing We rewritten this part.
Line 360-361: It is unclear what the bounding box should be selecting. No point has told us what to focus on. We now state that the focus in on the egg chamber in the bounding box. Additionally, it would be helpful to reference a zoomed in figure of the dialog boxes with entries highlighted for the next steps. We now provide instruction tutorial with screenshots.
Line 360: Are the coordinates obtained by mousing over the image? We now specify how to do it in the text.
Line 366-373: Again, referencing a zoomed in figure of the appropriate dialog box would be helpful. We specify it now.
Line 366-373: This line is not a method. What are blobs? Why is this step crucial? Perhaps more explanation is required here. We agree and changed it in the text with providing more explanation.
Line 368-369 could be left as a note, but the rest of this information seems it is important enough to be separated out of the Note section (insert Line 369-373 above line 368). Thank you, we corrected it as suggested.
Line 375: What is being done and what is its purpose? Is the ellipsoid drawn by the program? How are samples chosen? We explain now.
Line 377:Do you mean designated or desired rather than designed? To avoid confusion, we use the word 'desired' ellipsoid.
Line 381: See above comment for Line 377 Corrected to desired ellipsoid.
Line 392: See above comment for Line 377 Corrected to desired ellipsoid.
Line 400-401: What would the log file be used for? One can easily see the parameters in case of repetition. We explain why is that important.
Line 403: when is this necessary. Now we state when it is required.
Line 413-421: Again, it is unclear what this data is used for….what is the script for? What is the output? Again, a figure showing the dialog boxes would be helpful. We now explain.
Line 428-429: Either describe how this is done in PIV Lab, or merely state that manual analysis is not suitable for tissue scale images. We excluded this from our revised manuscript.
Line 485: Spelling-change cytoplasmatic to cytoplasmic, change "its" to "their" Ok, corrected.
Line 485-495: It think this figure would benefit from editing. Screenshots of dialog boxes are difficult to read. I may be better to create separate figures and reference these in the appropriate steps in the text. OK
Line 499: Is figure 2 being referenced in figure 3, or do you mean figure 3A? As in Figure 2, it may be better to create separate figures and enlarge screenshots of dialog boxes so they can be read. OK
Line 511-519: You reference AP axis, but it is not indicated in the movies or the legend. Thank you, we have now added that anterior is on the left in the movie.

5. Discussion

The discussion could benefit by expanding on the benefits of this method over other published methods for visualizing actin. At some point in the manuscript you need to discuss what the data is useful for. The discussion would be a great place to do this, with an example of wild-type vs mutant data.
We hope that we provide this now.

Line 579: Delete "and body's remains" as it is redundant with debris. Thank you, we have now deleted it.
Line 609-611: The wording is repetitive, there are grammar issues, and it is unclear. Re-word. Thank you, this is our mistake and now is corrected.
Line 617: change "your" to "the" OK
Line 617-619: Why can't the file be closed? This is a little confusing. Does ParticleStack.tif have to be open when correcting cell outlines in the original movie? We agree, this is our mistake after changes applied. We now deleted it.
Line 623- 627: True false positives need to be defined by statistics. We specify better.

Line 632-635: Perhaps this should be pointed out when discussing how to make the mask of cell outlines. We do now.
Line 656-667: It might be helpful to provide a schematic or picture illustrating an ideal ellipsoid and an ellipsoid that is not good to aid in explaining how this step affects blob identification. OK, we now provide this.
Lines 676-682: This info on the images is needed much earlier in the paper. We hope it is now.
Line 686-690: Expansion of the alternative uses to include specific examples - other organisms and tissue development would be appropriate and informative.
We now mention a few exmaples.

Reviewer #2:

Manuscript Summary:
Viktorinova et al describe methods for live-imaging the epithelial follicle cells of the Drosophila ovary such that apical or basal protein dynamics can be observed. This type of imaging has provided insight to actinomyosin dynamics and could be useful for further analysis in addition to characterizations of many other dynamic proteins. The title is appropriate; the abstract could be shortened but is also accurate. Overall the protocol is relatively

easy to follow, even for someone not used to the Fiji software, which is a plus. All materials and equipment are listed appropriately, and the procedure should be expected to lead the predicted outcomes. The sample images and movies provided in the protocol are impressive and useful.
We are very grateful for your valuable review and comments.

I have no major concerns, but I do have a number of comments and questions that would help make the manuscript maximally clear and useful, as follows:

Minor Concerns:
In the protocol description:
The authors should clarify that the starting flies need to be transgenics with fluorescently labeled protein(s) of interest (in this case, for myosin, MRLC-GFP). Since MRLC-GFP is used throughout, the protocol should include information on what this strain is (in text and figure legend) and a reference.
Thank you. We now provide information on MRLC::GFP in the text and legends as well as reference to the stocks used in the protocol.
It would also be helpful to comment on the larger blob of myosin in each cell- what is this thought to be?
We now state that the protocol focuses on the smaller Myosin II signals and users should avoid analysing the big ones. We would love to know what these big Myo-II signals are, however, we haven't found it yet. We think that it could be a sort of an adhesion type that links to the follicle epithelium to the surrounding extracellular matrix.

Are the flies fed fresh food each day (or just left with day one food) in step 1.2?
We now specify that flies should be kept in the same vials as prepared..

In my experience, the dissection strategy described in step 2.9 would lead to removing the abdomen completely. Is that the intention? We now changed this and specify that the abdomen should be pulled on the dorsal tip of the female abdomen in this point. However, it can sometimes happen that the whole abdomen gets separated from the thorax. Therefore, we added a note to this point and state what to do in such a case.
Are the ovaries falling out at the anterior of the abdomen? If not, the authors may want to explain how this is avoided or suggest holding at the top of the abdomen and not the thorax.
We added to hold one forcep on the thorax with the adjunct part of the abdomen. We believe that this will be clear from the video.

What is a cactus tool, and where can it be obtained?
We specify this in the Table of Materials.

TissueCellSegmentMovie-3.ijm script was a bit hard to find as it is embedded in text - more details on the website link would be helpful (eg, it is different than "tissue cell geometry stats"), as well as direction to the Supplementary Information given in Suppl.2.
We now provide detailed information.

Line 464 in the protocol itself. We included this information where to download the script into the protocol as you suggested.

I don't have the "surface manager" as an option under the "segmentation" plug-ins in Fiji, so I was unable to test the steps that required this. Where should I get this? Is it a Pc/mac problem? Perhaps the authors can advise in the protocol.
We now provide detailed instruction how to get this plugin and how to install it into Fiji.

I don't have "MultistackReg" from step 3.4.5 in my plugins? Can I used "correct 3D drift"?
Thank you for this information. Yes, you can use Correct 3D drift as well. We have added this information to the protocol as an alternative option. To obtain MultiStackReg, it is required to update Fiji as we describe in the protocol now.

The authors may want to explain best way to make submovies in step 3.5 without overlapping any information.
Thank you, we have now added the requested information.

More details needed for how to set up z-stack in step 4.1. Does "ca 60s" mean you should have 60 between acquisition of z-stacks? It is the time for one z-stack. We now state more in detail in this step.
How many microns is a typical required distance for the z-stack? We now specify the depth for a z-stack.

Steps 4.2.1 are a bit harder to follow. I think the authors acquire focal planes below the nurse cells but only use lateral information on the follicle cells, is that correct? We now improved this part. We first acquire the information for follicle cells and nurse cells in the z-stack. Then, we extract defined part of the surface based on the ellipsoid fitting and then from obtained surface projection select the layer of the interest.
See comments and questions for figure 3. Also, does the data to be inputted for 4.2.1.7 slice distance and projection information come from the confocal settings for z-stack? We recommend to use slice distance 1.

The majority of critical steps are highlighted, but authors may want to include making fresh media+insulin as a critical step. Also, some processing may not need to be listed as critical steps (for example the "Notes"), although they are probably needed for successful digital analysis.
Thank you for your kind comments. We provide additional information on this critical step in the dissection part.

Figure/Movie comments:
It would be helpful to have all egg chambers oriented the same way in figures, including schematics and movies, especially since in some there is no way to know the orientation as they are small sections.
Thank you for this point. We have rearranged our figures accordingly.

Figure 2 and legend- Overall this figure is extremely helpful, especially panels in F. It is not clear how helpful the screenshots are since they are so tiny and almost illegible. These may be better to include elsewhere and larger so they are more readable.
Thank you. We now provide larger figures.
Also, consider using "cytoplasmic" instead of "cytoplasmatic" in the figure. Thank you, we have changed it.
The legend should reference the corresponding movie so that the readers can see how the arrows in the analysis correspond to the dynamics of the movie.
Than you, we provide this now.

Figure 3 and legend- more detail here would be helpful. In B, where are the blobs and fit ellipsoid in these examples? We have now improved this part and replace by instruction tutorials.
Could the authors indicate this on the figure?
It seems like focal planes cuts through nurse cells for part of this - are the areas of nurse cells dropped out to capture lateral epithelial cells at this deeper focal plane, or are only very shallow optical sections used? These screenshots show lower planes of captured egg chamber in order to extract lateral follicle cells. We now state this in figure legends.
In fig3b, it looks like there is an intensity change in the very center - is that from nurse cells? Would that area be avoided in analysis? This is exactly why we have developed the surface extraction method. Myo-II expression is stronger at the apical side of follicle cells (i.e. closer to the nurse cells) than at their basal (i.e. outer) side. Therefore, this apical Myo-II information mixes with the basal Myo-II upon classical z-stack projection (see Fig. 1). Using the surface extraction, the apical Myo-II information is omitted.

For all movies, timestamps or total elapsed time needs to be specified on the movies or in the legends.
Thank you for this missing information. We have added it to all movies.

Some myosins seem to move across 2 cells. Why? Is this significant? Would they not be counted?
We agree that it is sometimes the case. We do not know, however, as to why is that/what is their origin and how come this at all. We are sorry that it is not clear from our protocol instruction (cytoplasmic, subcellular) and now we specifically point that these signals should be left out from the analysis.

Which way is movie 2 oriented?
Thank you for this missing information. We state now that anterior is on the left for both movies, 1 and 2.

In discussion section 2, it is suggested to have about 6 ovarioles or egg chambers per set up- are multiple egg chambers imaged at once? Or just one? Can a different egg chamber be visualized after one is used, or would it also be damaged from light exposure? This issue could be clarified.
We apologize for confusion if unclear in the text. We now state that only one egg chamber is imaged and discuss that there is no damage to other egg chambers in the same ovariole or other ovarioles in the SMI during imaging.

Other:
More explanation about why the processing steps are needed, especially the point of each digital manipulation, would be helpful for a novice.
For example, why is the surface manager needed?
Is this just to choose the subset of cells to analyze further?
If so, how many are needed? Since a number of manipulations are performed it is worth pointing out to a novice that original, unprocessed data should be kept separately.
We hope that through our new instruction it became clear now. The aim of this protocol is to convey how analyse actomyosin at the local and tissue scale. The dissection and imaging is leading towards it and may be even skipped as we now provide example data and test files.

How are the statistics obtained (as in fig 2D, steps 3.3.1-2) to be used?
Are these values needed later in the protocol? If it is just for quantification purposes, an example of that processed data (maybe in a graph) might make the utility clearer.
Since fat2 mutants are used to demonstrate proof of principal and differences observed in the mutant case, more background about this in the text would be helpful. The authors should reference their own paper as well as others on this, eg, Barlan (2017) Chen (2016). References should list all authors unless the journal style is

different.
We now provide a bit background in the figure legends what to observe in control and fat2 mutant egg chambers. However, we are limited here onto methodological part.

Discussion of future applications and limitations is quite good, however, it could be broadened to mention other protein analysis in egg chambers (in addition to actinomyosin applications in other tissues).
We now state that this protocol is suitable for actomyosin and similarly sized subcellular dynamic particles and mention other tissues that can be subject to this analysis.

Reviewer #3:

Manuscript Summary:
Viktorinova et al present a protocol for ex vivo culturing of Drosophila egg chambers and a pipeline for biological image analysis of actomyosin dynamics in the follicular epithelium. JoVE videos are particularly useful for manipulative techniques such as tissue micro-dissections, so this topic is appropriate, particularly because the preparation and ex vivo imaging of Drosophila egg chambers is used by a growing number of labs, but videos of this method are not readily available. An additional strength of the manuscript is that the protocol for tissue preparation and ex vivo culture is coupled with a detailed image analysis pipeline, an essential linkage for researchers that study the morphogenesis of the follicular epithelium, particularly cytoskeletal dynamics in the epithelium of the early egg chamber. However, in its current incarnation, the protocol is minimally detailed, often vague and unclear, and unlikely to lead to the described outcome, particularly in the hands of novices. Essential revisions are needed to render this protocol 1) discoverable: abstract does not adequately articulate the focus and usefulness of the protocol; 2) Informative: as written, readers have to read through the entirety of the protocol to know what data they will acquire; and 3) complete: essential details are missing for successful completion of the protocol. In the opinion of this reviewer, the key innovation to the tissue preparation portion of the protocol is the minimalist method to mount tissues for imaging. The authors of this manuscript could expand this section, as well as the time-lapse imaging section and the subsequent image analysis pipeline section, by reducing or eliminating details of the dissection and culture conditions portions. These portions of the tissue preparation protocol are elaborated more thoroughly in the cited 2007 Nature Protocols paper by Prasad et al., and an uncited 2014 Methods in Molecular Biology paper (Cetera, Llewellyn, and Horne-Badovinac, "Cultivation and Live Imaging of Drosophila Ovaries", Chapter 12 of "Drosophila: Methods and Protocols" in Methods in Mol. Biol. Vol. 148, Dahmann ed., pp. 215-226. The authors cite a less detailed 2014 paper by Cetera et al, in Nature Protocols.)
Thank you for your suggestions. We have now moved dissection and imaging section into a supplement and focus on the data analysis. We also cite the other protocol, although not suitable for this analysis, in the relevant place.

One major oversight in the protocol presentation is the absence of a description of the statistics that are calculated. "Press Statistics" is stated in several steps of the protocol. Without an explicit discussion of the statistical analysis performed, it is difficult to generalize this protocol to other applications, or to have confidence in the data generated for this application. This information is not visible in the screenshots in data processing steps shown the figures.
Thank you for this point. We improved this part and now added corresponding screenshots and explain what parameters ‘Statistics’ button provides in the instruction material.

Major Concerns:

----Are the title and abstract appropriate for this methods article?

The title is appropriate, but the abstract is not. The authors give a scientific description of the subject for their investigation, but do not provide specific information about the tissue preparation protocol and image analysis pipeline that are the actual subject of the methods described. They do not state the specific applications of their protocol beyond stating that it is useful for "in vitro live imaging" and analysis of "dynamic actomyosin machinery…at the cellular and tissue level."
We now provide a changed abstract that hopefully better reflects our protocol.

Rather than simply saying "cytoskeletal dynamics", the authors should include: 1) how actin and myosin are visualized in live fluorescence microscopy; and 2) The specific types of cellular and behaviors and cytoskeletal dynamics that can be measured with their protocol and analysis pipeline. It is not clear until late in the protocol that the image analysis pipeline
We now provide this information.

----Are there any other potential applications for the method/protocol the authors could discuss?

As written, the protocol is specific for imaging a very specific set of cell biological events during epithelium circumferential migration. The authors state that it is designed for detailed analysis of actomyosin machinery, but the image analysis protocol seems to be specific to periodic myosin localization into "blobs."

Thank you for this point. We now specify what myosin signals should be analysed.

The tissue preparation and imaging protocol appears to be optimized for imaging of pre-vitellogenic egg chambers (stages 1-8). Furthermore, the specific protocol for time-lapse imaging is adapted to view fast events over a very short time period (See Lines 187-188). This creates a limitation that should be presented early in the introduction, because many morphogenetic events in the egg chambers take periods greater than 1 hour. At some point in the protocol or discussion, the authors should discuss the pros and cons of changing the time-lapse specifications to encompass slower or longer events.
We completely agree that this was not clear in our first version. We now specify or the local cellular scale and for the tissue scale what time and resolution can be gained and what are limitations of these approaches.

There are many, widely accessible, transgenic reagents used to visualize actin and myosin; the authors should specify which reagents have worked in their hands, and particularly the genetic tools they visualized for the movies they show in the supplementary data, and which haven't if these have been encountered.
Thank you for this point. We have now specified what transgenic lines we have used.

At a minimum, authors should cite Spraklen et al., 2014, Dev Biol 393, 209, which critically assesses the utility of common expression drivers and actin labeling tools for in vivo studies of oogenesis.
Thank you. We added this citation to our text.

In contrast to potential limitations of the live imaging protocol, the image analysis pipeline, including cell segmentation, and the measurements of "statistics" such as cell area, cell shape, etc. could be useful to researchers studying later stages of egg chamber development. It would be important to state which parts of the ex vivo culture methods are restricted to use with inverted microscopes, and what parts of the image analysis pipeline can be used for time-lapse image data acquired using other culture and imaging conditions.
Thank you for this suggestion. We now specify what microscopes are needed for which part of the protocol. We have also corrected the statistics parameters and avoided unnecessary ones.

To summarize the comments in this section, it is not clear how adaptable this protocol will be. Assessing the adaptability for either the imaging or the image analysis pipeline will require additional notes and comments on the range for variation in parameters that are detailed under items 3 and 4, below.
We now state in the introduction and in the discussion how can this protocol be adapted and for what signals it can be used.

----Do you think the steps listed in the procedure would lead to the described outcome?

It seems unlikely, more on this is listed under the minor comments. One major issue is that the authors do not describe controls that could be used to check on proper performance of the technical steps in their protocol.
We now provide new instructions with screenshots and test files, which should hopefully make it easier for users to follow our protocol. Beside this, we are more specific about preparation and culturing of egg chambers.

The authors do not state the percentage of imaged egg chambers that yields a useable time-lapse data set, which will be needed to estimate the time needed for repeated rounds of the experiment.
Thank you for this point. We now included that the rate of successfully imaged undamaged egg chambers in the discussion part in troubleshooting for this section.

The authors don't explain how they avoid damaging egg chambers during imaging, nor do they summarize a list of specific precautions that are needed to avoid movement of the egg chambers in the culture medium.
We state in the dissection troubleshooting part what precautions and how avoid damaging of egg chambers.

The authors state that other published protocols introduce additional steps to limit egg chamber motion, but that the interventions compromise the egg chambers. However, this protocol does not clearly state how their methods avoid small movements that would impact the imaging session. It seems likely that the authors avoid slight movements of free egg chambers by imaging only one egg chamber during one time-lapse imaging session, due to inevitable slight motions caused by stage movements. If the authors do not encounter these limitations to their imaging sessions, they should explicitly say so, and then describe the important features of their protocol, stage, and stage control mechanisms, as well as the limits to distances travelled by the stage. If only one egg chamber is imaged per session, they should say so. Such a condition would necessitate access to the confocal or spinning disk microscope "on demand", rather that through advanced sign-up (a common condition for use of shared microscope systems).
Thank you for this point. We image early egg chambers that are mainly cross-linked in the ovariole, only occasionally there are egg chambers of stage 6-8 that are individually placed in the culturing medium. We image up to 10 egg chamber in one imaging (2h) session without changing media in a dish with egg chambers. We now describe this in the protocol and in the discussion troubleshooting part.

----Are any important steps missing from the procedure?

* Line 124, step 3, item 4: Other published protocols state that insulin must be dissolved in acidified water prior to addition to the Schneiders medium. They list both reagents in their list of materials (lines 444 and 445), but they do not state that the acidified water is specifically for the insulin solution. Within the protocol, they perform the pH measurement on the medium, prior to addition of the insulin. Do the authors intend for the insulin powder to be added directly to the medium. This is an example of details from other published protocols that are ambiguous in this protocol.
As we state in our Table of Materials, we use human insulin solution, no powder, i.e. there is no need to add acidified water into insulin. We add this insulin solution as described in the protocol, i.e. firstly after the Schneider medium mix and its pH adjustment is done. The volume of added insulin is ca. 0.1% of the total SMI volume and therefore does not change the final pH of the final used culturing medium.

* Lines 142-145: Step 2, items 11-12: The authors list a cactus tool in the Materials table, but do not say what it is used for. It would most likely be used for these steps, but no details are provided. Why use such a rare tool, available only to those with access to cactus spines? Why not use tungsten needles or other materials that can be ordered from microscopy suppliers?
Thank you. We now describe how to create cactus tool in the Table of Materials. You are right that one can use other similar tools if no cactus spines are available.

* Sections 3.2 (begin on line 202) and 4.2 (begin on line 339) on data processing: For each step in the analysis, it would be helpful to begin with an item for "Ensure fiji and the following plugins are downloaded", "Ensure that code is installed and working properly for _____", or "Use a computer with Matlab and the following toolboxes installed."
Thank you, we now guide the readers in detail and instruct them to download and open the corresponding script/code in indicated sections.

----Is there any additional information that would be useful to include?

* There are many points in the protocol that suggest that this pipeline is optimized for image data acquired with a 63X immersion objective, with at least 1.3 NA. This implies that other investigators who use a different objective might need to optimize settings differently. Is optimizing settings straightforward for each step, or will other investigators struggle to apply this protocol in a different setting?
Thank you for this point. We now state in the manuscript that it is not recommended to use other objectives with lower resolution and lower NA. We believe that this restriction should be no limitation for majority of scientist as such objectives can be easily purchased or are a state-of-the-art equipment of confocal microscopic settings.

* The authors state that images should be saved in .lsm or .czi formats, but it is not clear what these file formats are. Is .lsm proprietary to Zeiss microscopes? Do the authors mean that image data should be stained in the native file format for the microscope image acquisition software? If so, that would include other formats, such as Nikon's .nd2 format.
Thank you. Yes, you are right .lsm and .czi are Zeiss output formats. However, you can use any formats that can be opened in Fiji as now stated in our revised protocol.

* The intended purpose for some of the data analysis steps is not explained. "Bleach correction", introduced in line 204, is one example. A table or diagram that outlines the purpose of each step in the analysis pipeline would be very helpful, for example: Bleach correction does this; cell segmentation does this; surface extraction does this. Gaussian blur does this, a blob represents that. Such a table should include notes on critical parameter adjustment, if appropriate.
Thank you. We agree that this will be helpful and therefore, we now added this information to individual steps in the protocol.

Details on Vague or Problematic Steps of the Protocol:

Either in the abstract or in the introduction, the authors should inform readers that the protocol is only compatible with inverted microscopes. Similarly, the time limitation for the time-lapse imaging should be stated up front, so that investigators can assess whether the protocol can be used with their major equipment.
We agree and we hope it is clear in our revised manuscript.

------Table of materials needed lacks key information:
* The authors do not indicate that live imaging of cytoskeletal dynamics requires the use of fluorescently-labeled proteins. To use this protocol, investigators must have appropriate fly strains available, and these live materials are not mentioned at all.
Thank you for this point. We now added this information in the protocol and the Table of Materials.

* The authors do not list the MATLAB programming environment, nor do they list the MATLAB Statistics and Image Processing toolboxes.

We now decided to retract MatLab information as this is a preliminary outcome and may be misleading for readers.

The use of the Statistic analysis is described in step 3.3.2 lines 254 and 269, step 4.3 in line 416, "Critical Steps and Troubleshooting" lines 624 and 639, and Figure Legend 2 in line 491. The Image Processing toolbox needed to access the Particle Image Velocity" (PIV) analysis in step 4.4, line 429. On lines 698-699 they state that this is available in "the up-to-date" MATLAB version, but they don't give a version number. They do list a 2014 paper on MATLAB and the Statistics Toolbox in the reference list, but I could not find where this is cited in the text. It is not clear whether this reference also describes the algorithms for the PIV analysis.
We apologize for this confusion. As stated in the point above, we now retracted MatLab information. The reason is that our whole protocol in the manuscript is NOT based on the MatLab software, but on exclusively Fiji-based plugins. Hopefully, the removal of the MatLab program will dismiss this confusion.

* Line 452: Holder for cactus spine should be specified in more detail.
We provide this information in the Table of Materials.

-----Many steps in the described feature are vaguely described:

Many section headings in the protocol section are uninformative (i.e. "data processing"; "later analysis" "cellular scale" "tissue scale" (for the later two, the reader must do a lot of work to know precisely, how the authors are applying these terms. This needs to be spelled out in the introduction)
Thank you for this suggestion, we now changed and improved titles so that they are hopefully clear what the part will be about.

For reagent preparation, the authors should indicate final solution concentrations.
We now specify the concentration of our dye solutions. The other concentrations should be easy to follow.

Specific examples of vague statements in the protocol:
* Lines 126-127 should specify a period of hours during which the SMI is "good" instead of saying "it is possible to use the same SMI on the day of preparation" but not the next day. Does this mean the solution must be used within 8 hours? 16 hours?
Thank you for this point. We now added this information.

* Line 147: this description of an ovariole that lacks a muscular sheath is not sufficient. This is a key step of sample preparation and the authors give minimal instruction in terms of how to handle the ovarioles (they save this problem for the discussion) This should be clearer within the protocol. The statement also brings up the issue of similarity between this protocol and the protocol described in Cetera, Llewellyn, and Horne-Badovinac, "Cultivation and Live Imaging of Drosophila Ovaries", Chapter 12 of "Drosophila: Methods and Protocols" in Methods in Mol. Biol. V 148, Dahmann ed., pp. 215-226. The authors do not cite this paper, nor do they acknowledge any of the authors.
Thank you. We have changed it accordingly in the protocol. Due to the space limitation given by JoVE policies and also our initial intended focus of the protocol, we have now moved the dissection and imaging part of egg chambers into the supplement and focus only on the actomyosin analysis for both, local and tissue scales. We now also cite the mentioned paper in the relevant place. Their protocol, however, is not suitable for this analysis due to the final mounting of egg chambers and thus differs from our protocol.

* Line 151, Step 13. The authors do not state how much medium can be removed, they just state "avoid that the ovarioles dry out." Is there a point where you add more medium? If so, does additional dye need to be added as well?
Thank you. We state this now. There is no need to add dye as it firstly comes in the following step.

* Line 158, Step 14: The authors simply say to add dye, but they do not explain what CellMask or FM4-64 dyes stain, nor do they explain why these dyes must be used for the protocol. As one proceeds through the protocol, it becomes clear that all of the image analysis begins with segmentation, but the authors never mention in the abstract or the introduction that a cell membrane marker is essential if one wants to quantify cell level actin dynamics.
Thank you, you are right. We now corrected it and added this information.

Furthermore, they don't comment on whether commonly used membrane marking genetic tools such as shg::GFP or Indy::GFP could be used instead.
We agree that this could be better placed. We provide new version with this information.

* Lines 292-293, Step 3.5: These lines introduce step 3.5 as the most demanding part of the protocol. However, this section of the protocol encompasses only about 25 lines, with no explanation of some the terms, such as "time project" in line 298 and "signal line" in line 302.
Thank you, we provide now detailed protocol, which explain this.

* Line 266 and lines 378-373: "Find blobs": The authors do not explain what a blob is, either visually in the image nor in terms of the stained material it represents. Specific instructions are given, but the actual processes are not clear and leave a lot up to the interpretation of the reader.
Thank you, we now explain what is a blob and provide new detailed instruction for this part.

* Lines 335-337, Step 4.1: The authors state the time limit for acquisition of a z-stack, but they specify neither the number of optical sections nor the step size. They mention that you can image halfway through the egg, but do not state at which stage they do so, nor do they mention whether they routinely image this deeply for their tissue scale analysis?
We have improved this part and provide more information. However, how many optical sections or step size to choose is dependent on the microscopic setting of the user and may vary. We therefore avoid this information. Yes, it is required to image that deeply for the tissue scale due to the circumferential curvature of egg chambers. We explain this in the revised manuscript.

* Line 429, section 4.4: It is not clear what "(1.4)" refers to in this line. There is no step 1.4 in the protocol. Is this a typo?
We apologize for this confusion. This number refers to the PIV and we now removed this from the revised manuscript.

* Troubleshooting steps #2 and #3 do not have much substance as to how to solve the problems listed. Examples:
o Lines 593: "gently correct an unstable egg chamber" is a non-specific statement. Is the egg chamber manipulated, or is the microscope manipulated, and how?
Thank you. We agree that this is ambiguous and corrected it in the discussion part that it is done by moving the stage of the microscope.

o Line 602 - This sentence makes no sense in this context: "Count that high speed imaging results in fast bleaching of used fluorescent signals"
Thank you. We corrected it.

o Lines 610-611: "Reduce or increase the parameters based on the generated mask." How much should they be changed, and how is the mask assessed to know how to change them?
"it is crucial to create a clean mask" - what exactly does this mean? How does one ensure the mask is clean? What does it look like?
Thank you, you are right, we are really vague in our explanation. We apologize for this and provide a new version with more insights.

Minor Concerns:

Presumably the figures and movies are intended to indicate the anticipated results and desired outcomes, but these are not clearly explained. Figures are low resolution and cannot be read. Legends for Figures 1, 3, and 4 are insufficient to understand. The captions for the Movies discuss the overall biological interpretation, but do not relate them to the protocol, or provide sufficient information for a novice investigator to understand what they are seeing.
We are sorry to hear that you also had issues with figures. It must have happened by conversion of .psd files (600dpi, ca. 3000x5000pixels) into the wished ones by the journal. We now provide, hopefully, figures with decent resolution.

* Line 77 - Egg chambers are described as "cross-connected", which seems to imply a matrix of connected egg chambers, rather than a linear arrangement. This is stated again on line 143.
We removed the word “cross-connected’ and specify now that egg chambers are connected by stalk cells.

-----Are any important references missing and are the included references useful?

The included references are useful, but the cited references do not always appear to be the most relevant paper by the first author. For example, references #9, by Cetera et al 2016 is cited for biological observations of the same follicle cell migration, but the highly related methods paper by Cetera et al, 2016, published in Methods in Molecular Biology, is not cited (see above under #5, Line 147.
We don’t see a reason to cite this paper. This protocol is not suitable for live actomyosin network analysis presented in our manuscript.

The Spradling review from 1993 is cited for the presence of the muscle sheath, instead of either the primary literature or more recent research publications that used modern imaging methods imaging to study this structure.
We are sorry for this. We now added hopefully the right literature. Thank you for this improvement.

At least one other lab has performed related computational image analyses: Chen et al, 2016 (Cell Rep 15, 1125) used a different computational approach to derive a surface extraction from the entire egg chamber to evaluate events occuring during the circumferential rotation. The authors do not discuss the advantages and disadvantages of their methods relative to this, or any other approaches that have been used to evaluate dynamic events during circumferential migration.

Thank you. We understand your concerns and we now provide this citation and discuss what advantages our approach has over this one published recently by Chen et al.

1. Preparation of *Drosophila* females for ovary dissection

1.1. Sprinkle dry kitchen yeast on fresh standard fly food placed in small vials (ca. 15 mL). Place a 1:2 ratio of 1-2 day old males and females (max. 10 males:20 females) into the vial and let them feed for 2-3 more days to induce egg chamber production and oviposition in females.

1.2. Keep these flies at room temperature (23–25 °C) or in a 25 °C fly-specific incubator in the same vials as stated in 1.1.

1.3. Flip flies into a new vial with fresh food without kitchen yeast and prepare a Schneider mix as stated in the protocol step 2.

2. Preparation of Schneider mix for ovary dissection

2.1. Use sterile 50 mL conical tube and add 82% Schneider Medium: Add 17% Fetal Bovine Serum > Add 0.6% Streptomycin/Penicillin. Mix this Schneider mix (SM) well.

2.2. Remove small particles by using a filter (see Table of Materials). Adjust pH = 6.95-7.00 of the SM with 1N HCl. Store the SM at 4 °C for maximally 14 days.

2.3. Before dissection of *Drosophila* ovaries, add insulin to the SM to prepare fresh Schneider Medium with Insulin (SMI). The final insulin concentration should be 0.2 mg/mL. Allow the SMI to reach room temperature.

NOTE: Add insulin to the SM just before planned dissection. Never use older SMI than 8h stored at 4 °C.

2.4. Pipette 100 µL of the room-tempered SMI onto the prepared depression dissection glass and 100 µL onto the glass part of a dish at the dissection stereoscope.

3. Dissection of *Drosophila* ovaries

3.1. Anesthetize flies in the prepared vial from the protocol step 1 and place them onto a CO_2 supplied fly pad to immobilize them. Under the dissection stereoscope, select 4–6 females and leave them on the fly pad.

3.2. Use forceps to place one anesthetized female from the fly pad and place onto the SMI in the depression dissection glass.

3.3. Under the dissection binoculars (ca. 2x magnification) provided with a cold light, hold the female by the thorax and the adjunct part of the abdomen with one of the forceps and pull the abdomen cuticle (the dorsal part that is closer to the end tip of the abdomen) with the other forceps in the opposite direction.

NOTE: In case that the whole abdomen separates from the rest of the female body (thorax), discard the abdomen and dissect another female. However, once experienced with dissection of the ovaries, it is easy to identify and dissect ovaries also from the separated abdomen.

3.4. Ovaries will be pulled off with the other fly organs placed in the abdomen. Select ovaries, they look like two bundles of grapes and place them into the freshly prepared SMI on the dish (see the **Table of Materials**).

3.5. Hold gently the widest part of ovaries with one pair of forceps and hold one of the oldest egg chambers with the other forceps at this part. Pull out gently the oldest egg chamber out of ovaries. The string of egg chambers called ovariole should be automatically pulled out together with the oldest egg chamber. Should it not be the case, repeat this step with a slightly younger staged egg chambers.

NOTE: Pull always on the other side than egg chambers of your interest are placed in ovaries.

3.6. As ovarioles are covered with a contractile muscle sheet, check that dissected ovarioles are properly separated from the tip of ovaries and other ovarioles to ensure that muscle sheet was removed.

NOTE: Do not touch egg chambers of your interest with forceps to avoid damaging them.

3.7. Select six to seven separated ovarioles, which appear as a node-free string. By using the cactus tool, move them gently in the SMI to one side of the dish. Chose a side where a minimum of debris and old egg chambers are placed.

NOTE: If individual egg chambers of the interest (stages 6–8) are present in the SMI, include them too.

3.8. By using forceps, remove then all unwanted egg chambers together with debris, a dissection product from the SMI.

NOTE*:* If the SMI still appears dirty, pipette 2/3 of the SMI in the dish gently out. Do it on the other side than the egg chambers and ovarioles of your interest are placed in the dish. It is crucial to prevent egg chambers from drying out.

3.9. To visualize cell membranes in egg chambers, add 1 µL of the 1:100 membrane dye to the 100 µL SMI with the selected ovarioles in the dish about 10–15 mins before planned imaging.

NOTE*:* This estimated time is essential to allow the dye to penetrate into the egg chamber tissue. Optimization may be required for all other applications.

3.10. Mix gently with dissecting forceps. Avoid contact of ovarioles with the forceps though and cover the dish by a lid provided by the manufacturer.

4. **In vitro life imaging of actomyosin signals at the local cellular scale (up to 15 analyzed cells per an egg chamber)**

NOTE: This protocol step requires an inverted confocal microscope with a 63x water objective to obtain sufficient resolution of actomyosin signals, corresponding laser lines to image fluorescent tags/dyes, a camera to acquire time-lapse movies (TLMs) in various channels and a standard computer storage (up to ca. 70 MB per one TLM).

4.1. Immobilize cultured egg chambers and ovarioles.

4.1.1. Take the closed dish with six to seven ovarioles and free egg chambers to an inverted confocal microscope. Gently attach the dish to the specialized microscope holder that is designed for Petri dishes.

4.1.2. Open the lid and spread the selected ovarioles or older individual egg chambers (stages 6–8) with a cactus tool under 10x magnification and differential interference contrast (DIC) microscopic set up. Let the ovarioles settle down and make sure that they are substantially far from each other to avoid any potential motion generated by neighboring ovarioles.

4.1.3. Control egg chambers and ovarioles for rests of the muscle sheet and remove all ovarioles with muscle sheet from the SMI. Close the dish with its provided lid to avoid drying during microscopy session.

NOTE: Muscle sheet that covers egg chambers in ovarioles is contractile and a source of a potential undesirable movements of ovarioles. This is a crucial step as ovariole movements may lead to the focus loss during imaging.

4.2. Acquisition of TLMs for detailed actomyosin behavior

4.2.1. Focus the microscope with 10x objective and DIC on one selected ovariole and inspect again for presence of muscle sheet.

4.2.2. Find an egg chamber of the interest in the selected muscle-sheet-free ovariole. By using DIC settings, focus microscope into the middle of this egg chamber.

4.2.3. By using fluorescent reflectors, check for the appearance of cell membranes in the selected egg chamber, any strong dye staining point to a damage of the egg chamber. Do not image this egg chamber and search for other healthy one.

4.2.4. Set up acquisition parameters including corresponding laser lines, laser blocking filters, camera, etc. on the microscope. Follow providers instructions.

NOTE: Avoid strong laser power as it can damage/burn egg chambers. The damage of egg chambers can be observed as a sudden absence of epithelial rotation and in the worst case as a visible tissue damage during TLM acquisition. The correct settings must be tested in advance prior to TLM acquisition of selected egg chambers.

4.2.5. Change the 10x to a water 63x objective with numerical aperture (NA) of at least 1.3. Use water immersion to keep the light refraction similar to the used medium.

NOTE: A 63x objective provides sufficient resolution to observe subcellular actomyosin signals in egg chambers. Other objectives with lower resolution and lower NA will not give clear subcellular actomyosin signal that can be later comfortably analyzed.

4.2.6. Focus on the middle central area of the egg chamber and then on the most outer circumferential area of the egg chamber. Slightly lower the focus onto the surface of egg chambers with clear actomyosin signals.

4.2.7. Set up confocal imaging for single plane scans with time intervals of 6–12 s for 5–10 min to achieve short-time high-speed live imaging of the tissue surface of a selected egg chamber.

NOTE: No *z*-stack is required to acquire outer circumferential actomyosin behavior for ca. 5 cells in egg chambers of stage 1–5 and for up to 15 cells in egg chambers of stages 6–8. Before using the microscope, make sure that the microscopic table is well sprung/air-cushioned from the floor to reduce vibration that could affect imaging.

4.2.8. Save the desired TLMs.

NOTE: All file formats that can be opened as a time-lapse stack in Fiji (e.g., through **Bioformats**) can be used.

4.2.9. Note the orientation of a TLM, i.e., where is the anterior and posterior side of the imaged egg chamber. This information is required for further analysis.

NOTE: After imaging of one egg chamber, follow directly an acquisition of a TLM of another independent egg chamber in the same dish. With practice it should be possible to image up to 10 individual egg chambers of stages 1–8 (i.e., 10 acquired TLMs) within one imaging session of 2 h.

1. **Preparation of *Drosophila* females for ovary dissection**

1.1. Sprinkle dry kitchen yeast on fresh standard fly food placed in small vials (ca. 15 mL). Place a 1:2 ratio of 1-2 day old males and females (max. 10 males:20 females) into the vial and let them feed for 2-3 more days to induce egg chamber production and oviposition in females.

1.2. Keep these flies at room temperature (23–25 °C) or in a 25 °C fly-specific incubator in the same vials as stated in 1.1.

1.3. Flip flies into a new vial with fresh food without kitchen yeast and prepare a Schneider mix as stated in the protocol step 2.

2. **Preparation of Schneider mix for ovary dissection**

2.1. Use sterile 50 mL conical tube and add 82% Schneider Medium: Add 17% Fetal Bovine Serum > Add 0.6% Streptomycin/Penicillin. Mix this Schneider mix (SM) well.

2.2. Remove small particles by using a filter (see Table of Materials). Adjust pH = 6.95–7.00 of the SM with 1N HCl. Store the SM at 4 °C for maximally 14 days.

2.3. Before dissection of *Drosophila* ovaries, add insulin to the SM to prepare fresh Schneider Medium with Insulin (SMI). The final insulin concentration should be 0.2 mg/mL. Allow the SMI to reach room temperature.

NOTE: Add insulin to the SM just before planned dissection. Never use older SMI than 8 h stored at 4 °C.

2.4. Pipette 100 µL of the room-temperature SMI onto the prepared depression dissection glass and 100 µL onto the glass part of a dish at the dissection stereoscope.

3. Dissection of *Drosophila* ovaries

3.1. Anesthetize flies in the prepared vial from the protocol step 1 and place them onto a CO_2 supplied fly pad to immobilize them. Under the dissection stereoscope, select 4–6 females and leave them on the fly pad.

3.2. Use forceps to place one anesthetized female from the fly pad and place onto the SMI in the depression dissection glass.

3.3. Under the dissection binoculars (ca. 2x magnification) provided with a cold light, hold the female by the thorax and the adjunct part of the abdomen with one of the forceps and pull the abdomen cuticle (the dorsal part that is closer to the end tip of the abdomen) with the other forceps in the opposite direction.

NOTE: In case that the whole abdomen separates from the rest of the female body (thorax), discard the abdomen and dissect another female. However, once experienced with dissection of the ovaries, it is easy to identify and dissect ovaries also from the separated abdomen.

3.4. Ovaries will be pulled off with the other fly organs placed in the abdomen. Select ovaries, they look like two bundles of grapes and place them into the freshly prepared SMI on the dish (see Table of Materials).

3.5. Hold gently the widest part of ovaries with one pair of forceps and hold one of the oldest egg chambers with the other forceps at this part. Pull out gently the oldest egg chamber out of ovaries. The string of egg chambers called ovariole should be automatically pulled out together with the oldest egg chamber. Should it not be the case, repeat this step with a slightly younger staged egg chambers.

NOTE: Pull always on the other side than egg chambers of your interest are placed in ovaries.

3.6. As ovarioles are covered with a contractile muscle sheet, check that dissected ovarioles are properly separated from the tip of ovaries and other ovarioles to ensure that muscle sheet was removed.

NOTE: Do not touch egg chambers of your interest with forceps to avoid damaging them.

3.7. Select six to seven separated ovarioles, which appear as a node-free string. By using the cactus tool, move them gently in the SMI to one side of the dish. Chose a side where a minimum of debris and old egg chambers are placed.
NOTE: If individual egg chambers of the interest (stage 6-8) are present in the SMI, include them too.

3.8. By using forceps, remove then all unwanted egg chambers together with debris, a dissection product from the SMI.
NOTE*:* If the SMI still appears dirty, pipette 2/3 of the SMI in the dish gently out. Do it on the other side than the egg chambers and ovarioles of your interest are placed in the dish. It is crucial to prevent egg chambers from drying out.

3.9. To visualize cell membranes in egg chambers, add 1 µL of the 1:100 membrane dye to the 100 µL SMI with the selected ovarioles in the dish about 10-15 mins before planned imaging.
NOTE*:* This estimated time is essential to allow the dye to penetrate into the egg chamber tissue. Optimization may be required for all other applications.

3.10. Mix gently with dissecting forceps. Avoid contact of ovarioles with the forceps though and cover the dish by a lid provided by the manufacturer.

4. *In vitro* life imaging of actomyosin signals at the tissue scale (50–100 analyzed cells per an egg chamber) in curved tissues

NOTE: This protocol step requires a spinning disc microscope with inverted stage and a 40x water objective, corresponding laser lines to image fluorescent tags/dyes, a camera to acquire TLMs in various channels and larger computer storage (around a few GBs per one TLM).

4.1. Preparation of cultured egg chambers and ovarioles for in vitro life imaging

4.1.1. Prepare the sample as described in the protocol steps 1–3.

4.1.2. Install the dish with separated ovarioles onto the microscopic table of a spinning disc microscope.

4.2. Immobilization of cultured egg chambers and ovarioles

4.2.1. Take the closed dish with six to seven ovarioles and free egg chambers to an inverted spinning disk microscope. Gently attach the dish to the specialized microscope holder that is designed for Petri dishes.

4.2.2. Open the lid and spread the selected ovarioles or older individual egg chambers (stages 6–8) with a cactus tool under 10x magnification and differential interference contrast (DIC) microscope set up. Let the ovarioles settle down and make sure that they are substantially far from each other to avoid any potential motion generated by neighboring ovarioles.

4.2.3. Control egg chambers and ovarioles for resting of muscle sheet and remove all ovarioles with muscle sheet from the SMI. Close the dish with its provided lid to avoid drying during microscopy session.

NOTE: Muscle sheet that covers egg chambers in ovarioles is contractile and a source of a potential unwished movements of ovarioles. This is a crucial step as through ovariole movements the focus might be lost during imaging.

4.3. TLMs acquisition of tissue actomyosin behavior

4.3.1. Focus the microscope with 10x objective and DIC on one selected ovariole and inspect again for presence of muscle sheet.

4.3.2. Find an egg chamber of the interest in the selected muscle-sheet-free ovariole. By using DIC settings, focus microscope into the middle of this egg chamber.

4.3.3. By using fluorescent reflectors, check for the appearance of cell membranes in the selected egg chamber, any strong dye staining point to a damage of the egg chamber. Do not image this egg chamber and search for other healthy one.

4.3.4. Set up acquisition parameters including corresponding laser lines, laser blocking filters, camera, etc. on the microscope. Follow providers instructions.

NOTE: Avoid strong laser power as it can damage/burn egg chambers. The damage of egg chambers can be observed as a sudden absence of epithelial rotation and in the worst case as a visible tissue damage during TLM acquisition. The correct settings must be tested in advance prior to TLM acquisition of selected egg chambers.

4.3.5. Change to a water 40x objective with at least 1.3 NA and use a water immersion oil.

NOTE: This objective allows to image at least one half of an egg chamber with a reasonable spatial resolution of actomyosin signals.

4.3.6. Focus on the circumference of the selected egg chamber and set up a *z*-stack with 'First slice'. Move to the middle of the egg chamber and set up 'Last slice'. This allows scanning through at least one half of an egg chamber. Set the *z*-step around 1 μm or with at least with a sufficient overlap of acquired planes. Aim for the shortest possible acquisition time (if possible $\leq$ 60 s) for one *z*-stack within a TLM.

NOTE: The depth of an egg chamber of the stage 6-8 is ca. 35 μm. The time required to scan through at least one half of an egg chamber can be varied according to the defined *z*-step. In principle, it is possible to image more than one half of an egg chamber.

4.3.7. Interleave no time between individual *z*-stacks, i.e. each *z*-stack in a TLM should be acquired directly after the previous one is finished.

NOTE: One *z*-stack that is acquired in $\leq$ 60 s provides information on actomyosin signals approximately at each minute.

4.3.8. Acquire a 30-min-long TLM.

NOTE: Longer acquisition time for TLM with *z*-stacks taken every minute results in no egg chamber rotation. Actomyosin signals should be on no occasion analyzed in egg chambers that do not rotate. However, it is possible to set up longer intervals between imaging of individual z-stacks, which can prolong the acquisition time of rotating egg chambers once the frequency of actomyosin pulses/oscillations is understood.

4.3.9. Save acquired TLMs.

NOTE: All file formats that can be opened as a time-lapse stack in Fiji (e.g., through **Bioformats**) can be used.

4.3.10. Note the orientation of a TLM, i.e., where is the anterior and posterior side of an imaged egg chamber. This information is required for further analysis.

NOTE: After imaging of one egg chamber, follow directly an acquisition of a TLM of another independent egg chamber in the same dish. In order to obtain 30-min-long TLMs, with practice it should be possible to image up to 2–3 individual egg chambers of stages 1–8 within one imaging session of 2 h.

Test files:

ParticlesStack1.tif :
https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Sample_data/ParticlesStack1.tif

ParticlesStack2.tif:
https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Sample_data/ParticlesStack2.tif

TestMovie1.tif:
https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Sample_data/TestMovie1.tif

TestMovie1_RoiSet.zip:
https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Sample_data/TestMovie1_RoiSet.zip

TestMovie1_Submovie_1.tif:
https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Sample_data/TestMovie1_Submovie_1.tif

TestMovie1_bleach.tif:
https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Sample_data/TestMovie1_bleach.tif

TestMovie1_bleach_reg.tif:
https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Sample_data/TestMovie1_bleach_reg.tif

TestMovie2.czi (large file ~1GB):
https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Sample_data/TestMovie2.czi

TestMovie2_bleach.tif:

https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Sample_data/TestMovie2_bleach.tif

TestMovie2_extraction.tif:
https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Sample_data/TestMovie2_extraction.tif

TestMovie2_RoiSet.zip:
https://git.mpi-cbg.de/scicomp/viktorinova_et_al_actomyosin_dynamics/blob/master/Sample_data/TestMovie2_RoiSet.zip